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DOCTORAL THESIS

Contractile activity of the bladder urothelium and lamina propria

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Contractile activity of the bladder urothelium
and lamina propria

By

Christian Moro

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in total fulfilment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

The normal function of the urinary bladder is to store and void urine in a controlled manner. During the filling stage the bladder exhibits spontaneous non-voiding contractions yet the mechanisms underlying these contractions are unclear. The internal lining of the bladder (urothelium/lamina propria) is an important regulator of bladder function by its involvement in sensory mechanisms and via releasing chemical mediators. In addition, the urothelium/lamina propria also exhibits spontaneous contractions which are mediated by unknown mechanisms. This activity may influence contractions of the bladder and play an important role in bladder function. The present study aimed to investigate the spontaneous contractions of the urothelium/lamina propria to identify receptors which modulate the activity.

In the absence of any neuronal input, strips of urothelium/lamina propria developed spontaneous contractions with a frequency of 3.72 cycles min⁻¹ and an amplitude of 0.65g. The frequency and tension of contractions was increased by stimulation of muscarinic receptors and α_1 -adrenoceptors, and inhibited by β -adrenoceptor stimulation. Each of these receptor systems is a target for clinical therapies used to treat bladder dysfunction, and these results identify the urothelium/lamina propria as a potential site of action for these agents. Using RT-PCR all α_1 - and β -adrenoceptor subtypes were present at the mRNA level in the urothelium/lamina propria, whilst organ bath experiments with receptor subtype selective agonists and antagonists demonstrated that the main functional adrenoceptors in the tissue were the $\alpha_{1A/L}$ - and β_2 -adrenoceptors. Functional experiments also showed that nitric oxide donors decreased the rate of spontaneous contractions and inhibited responses to muscarinic receptor stimulation or electrical field stimulation. However, nitric oxide was not released spontaneously in response to stretch, EFS or muscarinic receptor stimulation. Activation of the nerves innervating the urothelium/lamina propria results in tissue contraction, yet the dominant neurotransmitter released does not activate muscarinic, adrenergic, or purinergic receptors. Upon removal of the urothelium the baseline frequency and tension of spontaneous contractions, and the response to muscarinic and β -adrenergic receptor activation remained unchanged. This identified the lamina propria as the layer responsible for the contractile activity.

In conclusion, the urothelium/lamina propria exhibits spontaneous contractile activity that may influence bladder activity. The rate of contraction was higher in the urothelium/lamina propria compared to the detrusor; however, it is possible that in diseased states of the bladder, these tissues may be more tightly coupled leading to lower urinary tract dysfunction. Therefore, the receptors within the urothelium/lamina propria present novel therapeutic targets for the treatment of bladder disorders.

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***PUBLICATIONS AND ABSTRACTS ARISING FROM THIS
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2012

DECLARATION

This thesis is submitted to Bond University in fulfilment of the requirements for the degree of Doctor of Philosophy. This thesis represents my own original work towards this research degree and contains no material which has been previously submitted for a degree or diploma at this University or other institution, except where due acknowledgement is made.



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Chapter 1

GENERAL INTRODUCTION

1.1 ANATOMY OF THE LOWER URINARY TRACT

1.1.1 *The lower urinary tract*

The lower urinary tract consists of the urinary bladder (Figure 1-1), the ureters, urethra, and prostate (in males). The umbilical, posterior and anterior ligaments stabilise the bladder through their attachments to the pubic and pelvic bones. Situated inferior to the peritoneum and posterior to the pubic symphysis, the bladder temporarily stores urine until it is convenient to void. It is a hollow organ with walls of smooth muscle, internally lined by an epithelial layer and externally protected by a serosal layer. Although it is capable of storing up to 1 litre of urine, the bladder holds an average maximum volume of 500mL during the normal filling stage.

The spherical shape of the bladder varies as it fills and empties. The pressure of its own contents and that of neighbouring organs affects the bladder's shape and position. During filling it changes from a tetrahedral shape and becomes pear-shaped, rising into the abdominal cavity. When empty the bladder is reduced in size and held entirely in the lower region of the pelvis.

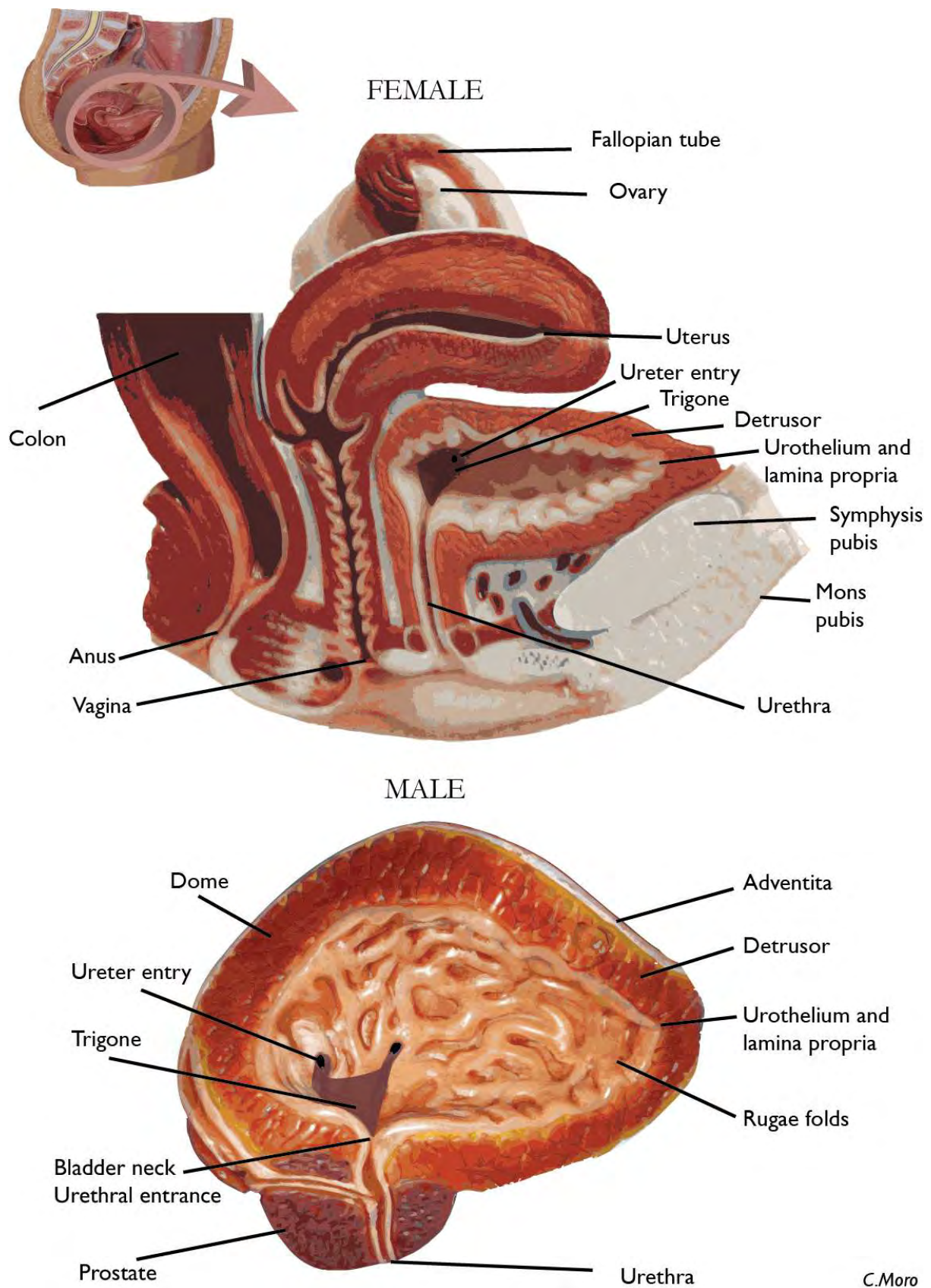


Figure 1-1: The location and regions of the female (top images) and male (bottom image) lower urinary tract.

The bladder is composed of three distinct regions (Figure 1-1) which include:

- The *trigone*: The triangular area of tissue at the base of the posterior wall. The slit-like openings of the ureters are approximately 2.5cm apart, and these openings form the top of the trigone, the inferior point of which is located just above the internal urethral sphincter. The epithelial layer here is smooth and the trigone acts as a funnel that assists in channelling urine into the urethra.
- The *bladder neck* and *urethral entrance*: The lower areas of the bladder that do not form the trigone.
- The *dome*: The main body of the bladder. The detrusor muscle is thickest in the dome and produces the strongest and most significant contractions during micturition.

The bladder wall is made up of several layers (Figure 1-2). The detrusor muscle forms the main bulk of the bladder and is externally protected by a layer of connective serosa, the adventitia. The detrusor consists of intermingled smooth muscle fibres arranged in a middle circular layer, with inner and outer longitudinal layers.

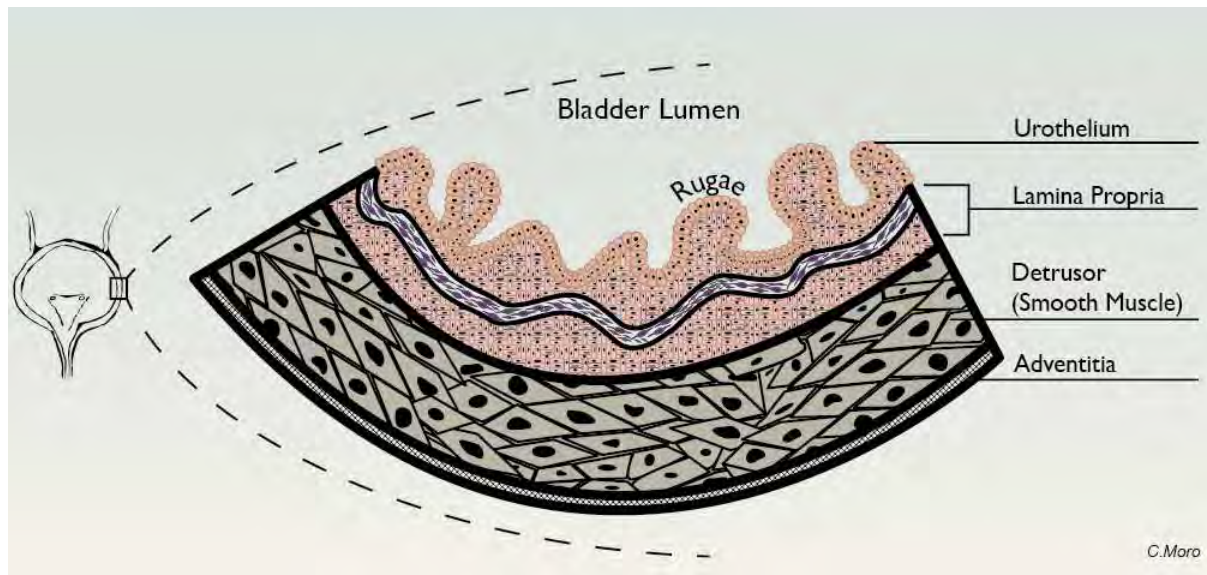


Figure 1-2: Illustration of the layers in the bladder. The black ‘cutout’ within the lamina propria layer has been enhanced to demonstrate the networks of interstitial cells within this cell layer.

Internally lining the bladder lumen are layers of specialised transitional epithelium, known as the urothelium (Figure 1-2). Underlying the urothelium is the lamina propria, which receives a rich innervation and blood supply (Dickson et al., 2006, Hossler and Monson, 1995, Hossler and Kao, 2007, Miodonski et al., 2001, Miodonski and Litwin, 1999). The terminology used when describing this layer varies. The layer of epithelial cells lining the inside of the bladder wall (and are in contact with the urine), is termed the *urothelium*. The underlying layers between these cells and the detrusor are termed the *lamina propria*. The urothelium/lamina propria is often referred to as the bladder *mucosa* or the *urothelium/suburothelium*.

Three layers of transitional epithelium comprise the urothelium (Figure 1-3). Each layer has morphologically distinct cell types due to the changes in cell shape and environment as the bladder fills and empties. The superficial layer contains “umbrella cells” that are able to flatten out to increase the surface area as the bladder fills (Figure 1-4). These large (diameters of 25-250 μm) hexagonal cells are interconnected by tight junctions (Apodaca,

2004, Lewis, 2000), which help prevent urine or dissolved chemicals in the bladder lumen contacting the underlying tissues. As the bladder empties, the umbrella cells reform back to their original shape, decreasing the surface area. When empty, the urothelial lining is arranged in folds called rugae that spread out as the bladder fills (Figure 1-5). These changes in surface area allow the bladder to enlarge, storing more urine without a significant rise in internal pressure.

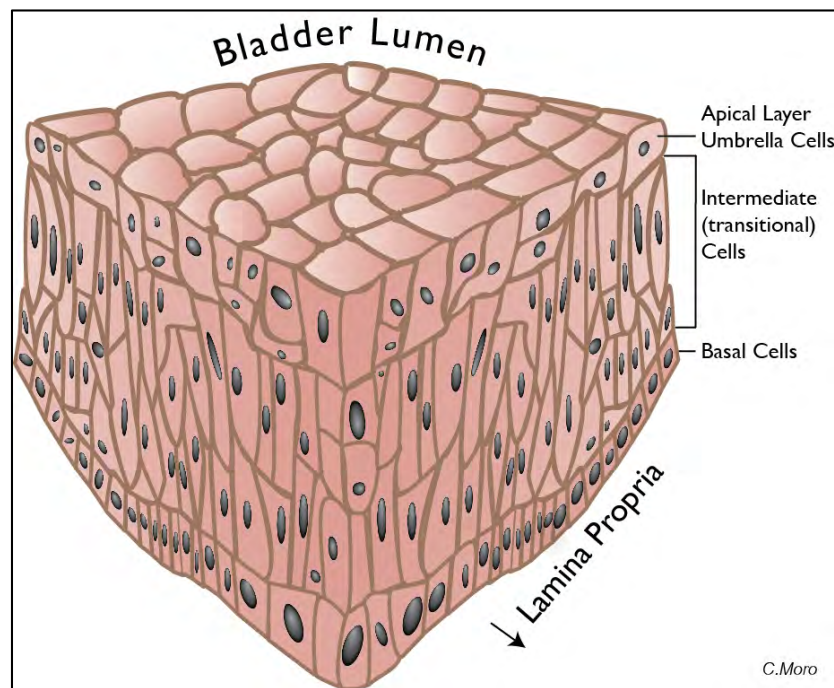


Figure 1-3: The layers of the urothelium. Image depicting the umbrella cells, intermediate layer and basal layer.

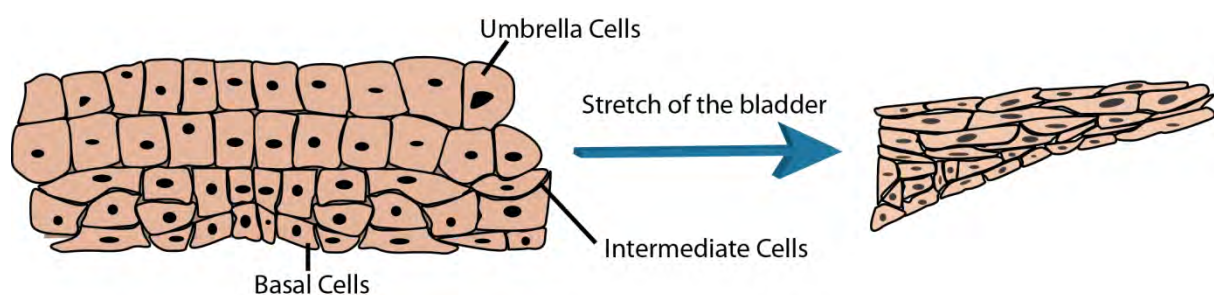


Figure 1-4: Depiction of the transitional nature of the bladder umbrella cells as a result of stretching as the bladder fills. These cells are required to maintain their protective coatings and tight-junctions during the shape transformations.

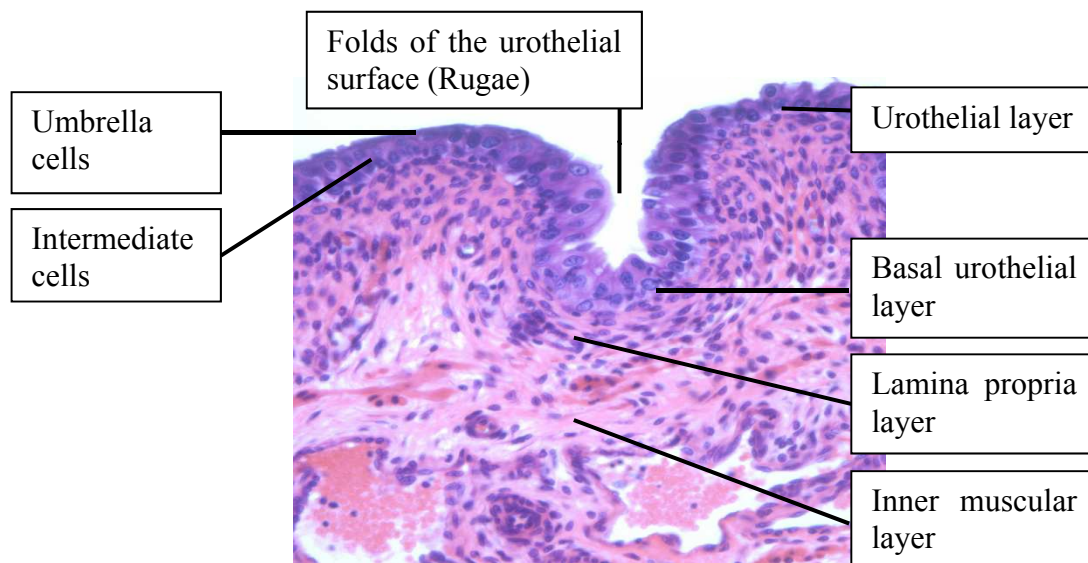


Figure 1-5: Haematoxylin and eosin stain of a transverse section of the bladder. The apical layers of the bladder, including the urothelial and lamina propria layers are labelled (Magnification: 100x).

1.1.2 The urethra and ureters

Urine is expelled from the bladder and leaves the body through the urethra. The urethra is a hollow tube with thick and elastic walls. It commences at the internal urethral sphincter, inferior to the bladder, and runs through the external urethral sphincter to the external orifice. It is composed of smooth muscle which comprises an outer longitudinal layer, a circular layer and an inner longitudinal layer (Dass et al., 2001). As a circular and striated band of skeletal muscle, the external urethral sphincter is under voluntary control and must be relaxed in order to permit the outlet of urine. The urethra varies in size, length and strength between males and females. In males, the urethra is longer and passes through the centre of the prostate gland (Figure 1-1).

Urine is filtered by each kidney into the urinary bladder through the ureters. These 30 cm long hollow tubes run from the renal pelvis and descend behind the peritoneum. The exact paths taken differ between males and females, due to the position of the reproductive organs (Figure 1-1). The ureters enter the lower side of the bladder through slit-shaped holes. Backflow of urine is prevented as the increase in bladder pressure causes the ureteral openings to close. The ureters facilitate the flow of urine from the kidneys to the bladder through peristaltic waves, induced within its walls. This peristaltic action is enhanced in frequency and strength if there are increases in the volume of urine released from the kidneys (Osman et al., 2009).

1.1.3 Barrier function of the urothelium

The most important role of the urothelium is maintaining an intact barrier to protect the bladder throughout the filling and voiding stages. The umbrella cells maintain tight junctions between adjacent cells which prevent urine or other chemicals (urea, protons and dissolved excreted substances) in the bladder lumen from contacting the underlying tissues (Peter, 1978). The surface of these cells is also protected by a crystalline protein layer which forms hexagonal plaques called uroplakins (Lewis, 2000, Apodaca, 2004, Wang et al., 2003, Tammela et al., 1993, Sun, 2006, Parsons et al., 1990). These plaques develop a unique permeability barrier which not only prevents nearly all normally permeable substances from passing through the apical urothelial surface, but also stabilises and protects the umbrella cells during bladder wall stretching (Hu et al., 2002, Negrete et al., 1996, Southgate et al., 1994).

The umbrella cells are further protected by their ability to produce a glycosaminoglycan (GAG) layer created from sulfated polysaccharide glycosaminoglycans (Romih et al., 2005, Parsons et al., 1979). The GAG layer consists of strings of proteoglycans composed of chondroitin sulfate and sodium hyaluronate. These molecules extend from the apical surface of the umbrella cells (Figure 1-6) and intertwine to act as a mucous-like barrier (Hurst and Zebrowski, 1994, Grist and Chakraborty, 1994, Nickel and Cornish, 1994). This layer binds to water molecules and forms an insulating layer between dissolved chemicals in the bladder lumen and the umbrella cells, further preventing damage to the urothelium and underlying tissues.

During the filling stage these protective layers are configured to avoid damage from the transitional changes in shape undergone by the umbrella cells. The umbrella cells in particular have complex mechanisms in place that allow for the internalisation and subsequent reinsertion of plaque membranes. This is achieved through the endocytosis and subsequent exocytosis of uroplakins (Apodaca, 2002, Truschel et al., 2002). These mechanisms allow the urothelium to maintain a highly specialised and effective protective role throughout the filling and voiding stages of the micturition cycle which is unique to the bladder.

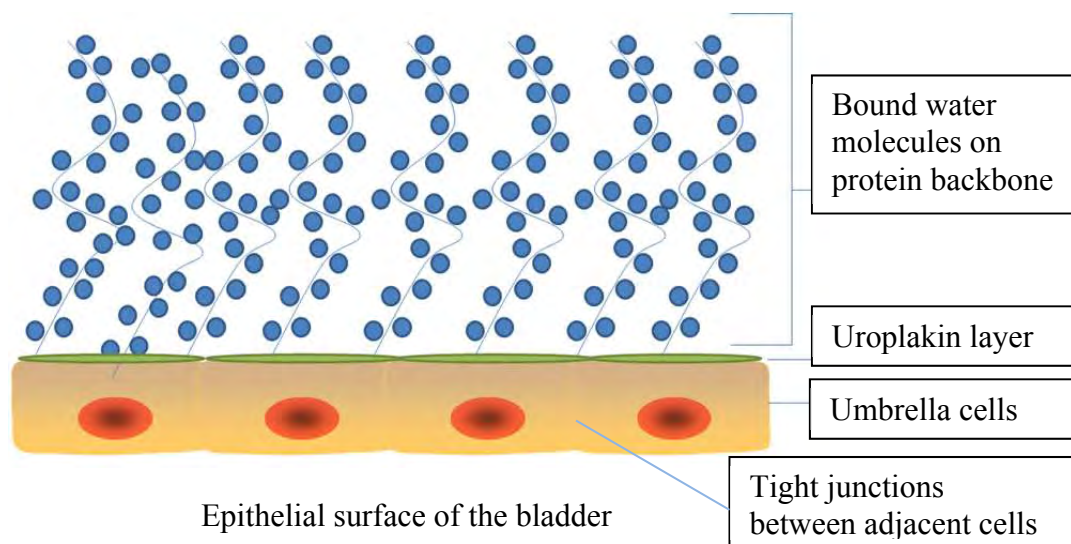


Figure 1-6: The apical urothelial layer. The figure demonstrates the urothelial cells, the protein backbone composed of sulfated polysaccharide glycosaminoglycans and the uroplakin layer.

1.2 INTERSTITIAL CELLS

The layer between the urothelial cells and the detrusor smooth muscle is termed the lamina propria and this region contains a diverse collection of cells and tissues. These include connective tissue, blood vessels, and afferent nerve fibres; as well as a range of cells types including interstitial cells and fibroblasts (Johnston et al., 2010, McCloskey, 2010, Rasmussen et al., 2009, Wiseman et al., 2003, Yu et al., 2011b, Woodman et al., 2011). Additionally, a range of immune cells are also present in the lamina propria, such as mast cells and dendritic cells (Christmas and Rode, 1991, Gardiner et al., 1986, Kummer et al., 2007, Yu and Hill, 2011). However, of most interest are the networks of interstitial cells, which are particularly evident directly below the urothelium (Wiseman et al., 2002, Blyweert et al., 2004, Wu et al., 2004, Kuijpers et al., 2007, Roosen et al., 2009).

The interstitial cells in the lamina propria have also been termed myofibroblasts (Sadananda et al., 2008, Fry et al., 2007) or interstitial cells of Cajal -like cells (McCloskey et al., 2009, Sui et al., 2002). There is considerable controversy and debate regarding the naming convention for these cells, yet they will henceforth be referred to as “interstitial cells”. Due to their appearance and location (Figure 1-7), the bladder’s interstitial cells were thought to be similar to the interstitial cells of Cajal in the gut, which act as pacemakers in the intestinal mucosa, generating peristaltic contractions (Sanders, 1996, Hirst and Ward, 2003, Gillespie et al., 2004). The lamina propria interstitial cells share some similarities to the ICC cells in the gut, such as their unique position under the basal cell layers in the lamina propria, and their similar immunohistochemical staining for c-kit, connexin-43 and vimentin, although individually, these markers are not specific to the cells (Gevaert et al., 2011, Johnston et al., 2010, van der AA et al., 2004, Cheng et al., 2011b, Kim et al., 2011). However, their

localisation in the bladder lamina propria, adjacent cell types, and potential functions in mediating bladder activity sets them apart from those in the intestinal tract.

There is evidence to suggest the role of the lamina propria interstitial cells as regulators of bladder spontaneous activity (Fry et al., 2004b, Montgomery and Fry, 1992, Sui et al., 2008). This activity is thought to be mediated by electrical and calcium transients across the lamina propria layer (Fry et al., 2012, Hashitani et al., 2004a) and the involvement of interstitial cells presents a novel target for its modulation (Ikeda and Kanai, 2008). Although interstitial cells in the lamina propria may have the potential to regulate the phasic contractile activity, their exact role is unclear (Wiseman et al., 2003, Fry et al., 2004a, Gillespie et al., 2006b). However, it is possible that these cells facilitate spontaneous activity through modulating the amplitude or acting as pacemakers (Kubota et al., 2011).

Historically, the presence of interstitial cells was determined through their histological structure (Gabbiani et al., 1971, Eyden, 1993) along with the presence of smooth muscle actin (Powell et al., 1999). It has been noted that past identifications of bladder interstitial cells had not conclusively shown the fibronexus (Eyden, 2009) or the full complement of organelles, which are common identifying features for “myofibroblasts” using contemporary techniques (Gabbiani et al., 1971, Ryan et al., 1974). Bladder urothelial/lamina propria interstitial cells are elongated spindle-shaped (Cheng et al., 2011b), and contain a rough endoplasmic reticulum, actin filaments and fibronectin fibrils (Drake et al., 2006). In addition, they have been shown to exhibit a basal lamina, which is usually lacking in fibroblasts. These types of differences have resulted in controversy over the precise name to describe these cells, and whether they fit the exact description of a ‘myofibroblast’ (Drake et

al., 2006). A depiction of a typical lamina propria interstitial cell, with its characteristic shape, organelles and filaments is illustrated in Figure 1-8.

The detrusor smooth muscle also contains interstitial cells, although they are morphologically different from those in the lamina propria. Two types of these c-kit positive cells are found in this muscle layer: one that has a stellate shape, similar to those seen in the lamina propria; and another with an elongated shape containing a number of lateral branches (Drake et al., 2003c, McCloskey, 2010, Brading and McCloskey, 2005). While the lamina propria interstitial cells form networks through gap junctions (Sui et al., 2002) and specific innervations (Wiseman et al., 2003), the detrusor interstitial cells are not networked with each other, yet appear to be present along the periphery of muscle bundles and close to innervations (McCloskey et al., 2009). This location may present them with a role in modulating neuronal input to the muscle cells.

It has been hypothesised that the detrusor interstitial cells might generate and modulate bladder spontaneous activity (Lagou et al., 2006, Brading, 2006). However, not all reviews agree with the minimal evidence that supports a pacemaking role (McHale et al., 2006b). Of particular interest is the lack of pacemaking activity arising from these cells when carefully observed using microelectrodes and intracellular calcium imaging (Hashitani et al., 2004b). It is also possible that the smooth muscle cells in the lower urinary tract have their own excitability, and as such may not be reliant upon pacemaking interstitial cells (Hashitani, 2006). Nonetheless, there is a clear lack of knowledge or research supporting a role for the interstitial cells in the detrusor, yet their lack of cell networks and close association with nerve fibres suggests that they are unlikely to be involved in the endogenous coordination and generation of spontaneous activity.

There is a rich network of capillaries and neuronal innervations to the lamina propria (Birder et al., 2010b), which may facilitate an important and active role for this region. There is experimental evidence supporting the hypothesis that the interstitial cells in the lamina propria can regulate contractile activity. For example, the interstitial cells respond to agonist activation, such as purinergic stimulation. This has been observed in intact guinea-pig bladder preparations where measurements of interstitial cell activity (isometric tension, intracellular calcium, and membrane current) has been increased in studies using the purinergic agonist UTP, which is known to excite interstitial cells in the lamina propria yet not the detrusor muscle itself (Fry et al., 2009, Sui et al., 2008). This purinergic receptor (P2Y) activation is also thought to potentiate bladder activity through direct stimulation of receptors on the interstitial cells (Wu et al., 2004, Cheng et al., 2011a). An individual receptor subtype (P2Y₆) was also identified as the most likely receptor on these cells (Sui et al., 2006). This is an important discovery as it demonstrates the potential to specifically modulate the interstitial cell activity within the lamina propria.

The lamina propria interstitial cells respond to purinergic stimulation by generating calcium transients and activating chloride currents (Sui et al., 2004). If two isolated interstitial cells are close together, each cell demonstrates an enhanced response to ATP. This is thought to be through a mechanism involving membrane proteins, such as cadherin-11 (Smet et al., 1996b) and investigations are still underway to ascertain the exact mechanism underlying this phenomenon (Fry et al., 2009). Nonetheless, the fact that increased urothelial ATP can contribute to afferent sensitisation via interstitial cells is potential justification for the importance of researching clinical purinergic antagonists, in particular for the treatment of abnormal sensory activity in the bladder. In addition, increased research into the activity of

the lamina propria interstitial cells may identify novel mechanisms of bladder modulation. The receptors which are expressed and functional on the interstitial cells may present new targets for therapeutic treatments in bladder diseases.

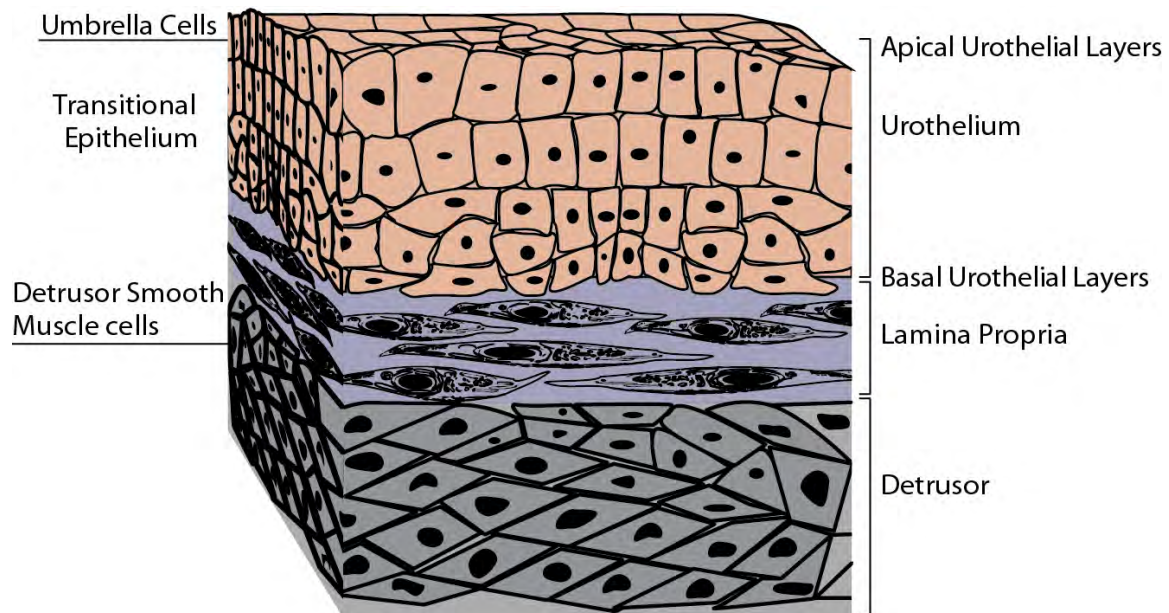


Figure 1-7: A section of the wall from the dome of the bladder. Image demonstrates the location of the network of interstitial cells within the lamina propria. This layer is situated between the urothelial cells and the underlying detrusor smooth muscle.

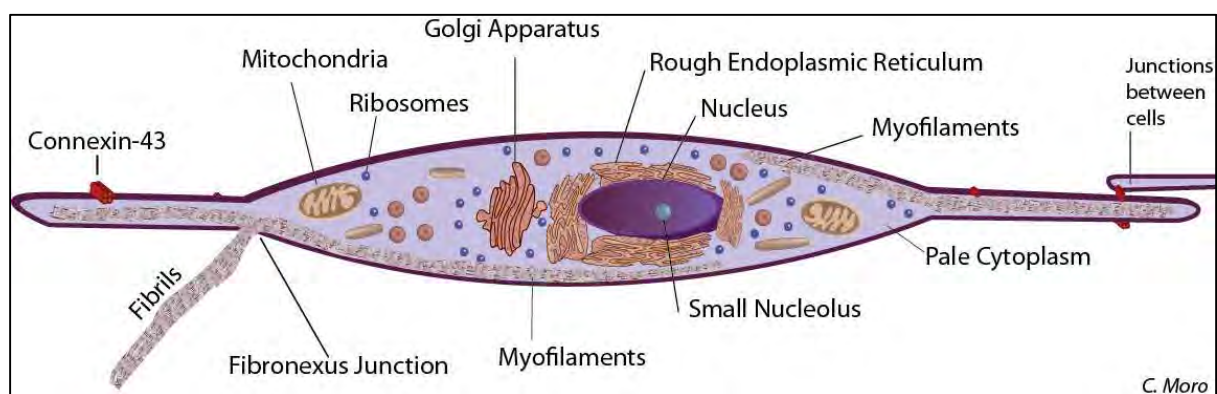


Figure 1-8: Image depicting a spindle-shaped bladder interstitial cell found in the lamina propria containing the characteristic histological and structural features.

1.3 NEUROPHYSIOLOGY OF THE LOWER URINARY TRACT

The bladder stores and subsequently releases urine in a controlled manner, which is part voluntary and part involuntary. Both the somatic and autonomic nervous systems innervate the lower urinary tract and neuronal control during filling and voiding requires coordination between the parasympathetic, sympathetic and somatic nerves.

1.3.1 The micturition cycle

The micturition cycle comprises the filling/storage phase followed by the subsequent voiding phase. The process of transferring between these two phases involves both the somatic and autonomic nervous system and is co-ordinated and under voluntary control in the adult, but is involuntarily controlled by an entirely spinal reflex in infants (de Groat, 1997, de Groat, 1993). Bladder activity is regulated through an area in the brain stem called the pontine micturition centre (Figure 1-9). The hypothalamus, frontal cortex and cingulate gyrus areas of the brain are also involved in bladder functions including the voluntary control of urination, awareness of bladder fullness and relaxation of the external sphincter (Blok et al., 1997b, Fowler et al., 2008).

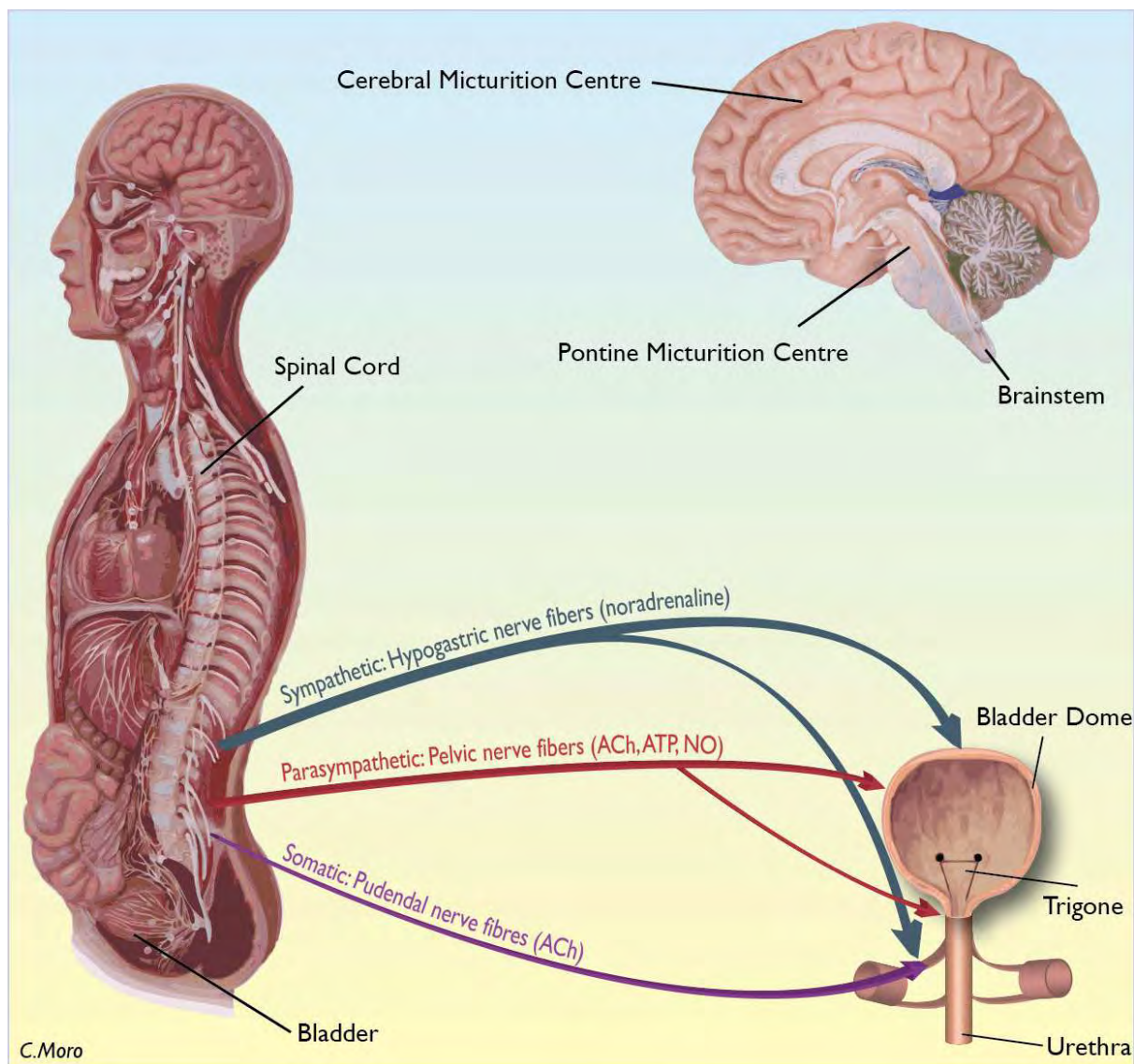


Figure 1-9: Innervation of the bladder. Diagram depicting the location of the supraspinal micturition centres (top right image) and the main neuronal innervations to the bladder (lower images). The co-ordination of the micturition cycle is regulated within the pontine micturition centre and signals are transmitted from the spinal cord via sympathetic, parasympathetic and somatic nerves. The parasympathetic innervation releases acetylcholine, ATP and nitric oxide. The hypogastric nerves contain sympathetic afferent and efferent nerves. Image collated from a range of sources including: (de Groat, 2006, Ford et al., 2006, Yoshimura et al., 2008b, Blok et al., 1997a, Andersson and Arner, 2004).

1.3.2 *Efferent innervation*

Bladder efferent nerves are known to be important in the normal regulation of the micturition cycle (De Groat, 1975, de Groat and Ryall, 1969, Iggo, 1955, Yoshimura et al., 2008b). Therefore, a clear knowledge of the mechanisms involved in neuronal control has greatly assisted in the understanding of bladder activity (Kanai and Andersson, 2010). Bladder emptying can be induced through stimulation of the spinal cord roots, identifying the importance of these lower urinary tract innervations (Brindley, 1995, Brindley, 1977). Both the sympathetic and parasympathetic nerves travel to the bladder via the pelvic plexus, and subsequently innervate the urethra, bladder dome and bladder neck (Fletcher and Bradley, 1978, Ek et al., 1977b).

There are three neuronal pathways from the spinal cord that innervate the lower urinary tract, the hypogastric, pelvic and pudendal pathways (de Groat and Ryall, 1969, de Groat et al., 1981, Morgan et al., 1993, de Groat, 2006, Ford et al., 2006, Yoshimura et al., 2008b). The pelvic nerve contains parasympathetic nerve fibres which release ATP, acetylcholine and nitric oxide, and extends from an area the spinal cord at the S2-S3 region (Thor et al., 1989). The pudendal is a somatic nerve which innervates the external urethral sphincter and pelvic floor muscles (de Groat et al., 2001, de Groat, 2006). The hypogastric nerve is a sympathetic nerve which leaves the spinal cord from the T12-L2 region. This nerve controls the tissue by releasing noradrenaline to act on the detrusor's β -adrenoceptors causing relaxation, or on urethral α -adrenoceptors to cause contraction. Ganglia in the bladder detrusor express enzymes for tyrosine hydroxylase (Smet et al., 1996a) as well a large proportion of neurons immunoreactive for human vesicular acetylcholine transporter (Dixon et al., 1999). In addition, the sympathetic innervation and ganglia in the lamina propria are also

immunoreactive for tyrosine hydroxylase (Dixon et al., 1999, Jen et al., 1995). The presence of this enzyme may give these lamina propria neurons the ability to produce noradrenaline, which would act upon the α - or β -adrenoceptors expressed here. However, the functional relevance for these receptors in the dome is still not clear.

1.3.3 Afferent innervation

Two types of afferent nerves are found in the bladder, the A-delta nerve fibres (A δ -nerves) and the C-fibres. The cell bodies of these afferent nerves are contained in the lower lumbar and sacral dorsal root ganglia (Kanai and Andersson, 2010) and transmit information to the brain as the bladder is stretched during the filling stage (de Groat et al., 1981). Bladder afferent fibres contain a range of neuropeptides, including substance P, neurokinin A, neurokinin B, calcitonin gene related peptide, cholecystokinin and enkephalins (Donovan et al., 1983, Vizzard, 2000, Vizzard, 2001, Keast, 1991, Arms and Vizzard, 2011).

The A δ -fibers are located in the detrusor smooth muscle and contain relatively few peptide neurotransmitters. They are thinly myelinated sensory fibres which can quickly transmit signals from the bladder to the spinal cord (Wyndaele, 2010). These A δ -fibres are activated by relatively low thresholds, such as increases in bladder volume or detrusor contractile activity (Iggo, 1955, Birder et al., 2010a). These fibres are therefore involved in the perception of bladder volume during the filling stage of the micturition cycle.

C-fibre afferents also innervate the detrusor. These nerve fibres, with their characteristic sensitivity to the agonist capsaicin, are unmyelinated nerve cells, less sensitive to distension than the A δ -fibres (Kawatani et al., 1986, Maggi et al., 1989, Bahns et al., 1987, Mazieres et

al., 1998, Fowler et al., 1994, de Groat et al., 1990, de Groat et al., 2006, Andersson and Hedlund, 2002, Birder et al., 2010a, Habler et al., 1990). There is little information on the role of these high-threshold C-fibre afferents in normal bladder function, although they appear to generate reflex contractions during the filling stage in some animal models (de Groat et al., 1990, Yoshimura and de Groat, 1997, Cheng et al., 1999). However, this involvement is not clear and as such these nerves are not thought to be responsible for neuronal control of the normal micturition cycle.

C-fibre afferents are activated in response to diseases of the bladder such as inflammation or overdistension (Wyndaele, 2010, Janig and Koltzenburg, 1990). This suggests that the C-fibre afferents may operate a mechanosensitive role, transmitting signals to the spinal cord during periods of inflammation or injury to the bladder. They may also provide the additional neuronal sensitivity, such as increased urgency and pain, observed in inflammatory bladder diseases (Habler et al., 1990). Additionally, abnormal regulation of this neuronal system may be functionally involved in stimulating the increased contractions seen in detrusor overactivity. Both afferent and efferent nerves in the bladder do not only innervate the detrusor muscle, but also extend into the lamina propria and come in close proximity to the interstitial cells, assumingly influencing their function (Wu et al., 2004, Sui et al., 2006, Ford et al., 2006, Kanai and Andersson, 2010, Gillespie et al., 2004, Hashitani, 2006, Yoshimura, 2007).

1.3.4 The filling and storage phase

The empty bladder increases in size as it fills, allowing up to 1L of urine storage. During the filling stage there is no significant increase in intravesical pressure due to a relaxation of

smooth muscle. As the bladder enlarges, the internal rugae of the urothelium disappear and the umbrella cells alter their shape and position to accommodate the increasing size and length of the bladder wall (Lazzeri, 2006). Low level firing of afferent nerve fibres results in the release of noradrenaline from the sympathetic nerves, which relaxes the smooth muscle in the bladder dome via activation of detrusor β -adrenoceptors (de Groat, 2006). At the same time noradrenaline released in the urethra activates the nearby urethral α -adrenoceptors and causes contraction (Fry et al., 2010). The release of noradrenaline is sensitive to stretch and as the bladder fills more noradrenaline is released, further stimulating detrusor relaxation. Other mediators are also involved in maintaining continence by facilitating the closure of the urethra and bladder outlet region, such as prostanoids, tachykinins (Andersson and Hedlund, 2002) and ATP, which contracts smooth muscle and helps maintain urethral closure (de Groat and Yoshimura, 2001). In males, sympathetic nerves also activate the α_1 -adrenoreceptors on the prostate smooth muscle which increases outlet resistance and assists in maintaining continence (Chapple et al., 1989).

1.3.5 The voiding phase

A neurogenic on-off circuit between the urinary bladder and urethral outlet causes the change from the filling/storage phase, to the voiding phase (Fowler et al., 2008). The increased intravesical volume as the bladder fills enhances activity in afferent and sympathetic nerves up to a critical threshold at which the voiding reflex is initiated. This afferent activity is relayed to the periaqueductal gray area in the pons which then transmits the signal to the pontine micturition centre and commences the voiding process (Sullivan and Yalla, 2002). When this occurs, the sympathetic and somatic pathways are inhibited and the parasympathetic innervation is activated (de Groat et al., 1997). These parasympathetic

nerves release acetylcholine (Figure 1-9) which activates muscarinic receptors on the detrusor and initiates contraction (Nausch et al., 2010). At the same time, ATP is released which acts upon detrusor muscle P2X₁-receptors to enhance contraction (Burnstock, 2001b, Heppner et al., 2009, Layne et al., 2010). Parasympathetic nerves also release nitric oxide to relax the urethra and bladder outlet region (Andersson and Arner, 2004). These processes facilitate the voiding phase of the micturition cycle, causing relaxation of the urethra and bladder outlet, contraction of the detrusor and voiding (Chancellor and Yoshimura, 2002).

1.4 SPONTANEOUS ACTIVITY

Many smooth muscle organs in the body exhibit some form of spontaneous rhythmic activity. These include the uterus, gastrointestinal tract, gallbladder, urethra, prostate and vas deferens. The common function of this spontaneous activity is to provide motility in these muscles, such as the ejection of fluids from the prostate or peristaltic contractions in the intestine. As a result the mechanical consequences of the activity vary between tissues, from rapid phasic contractions to sustained contracture (McHale et al., 2006a). The contractions are not necessarily related to input from nerves innervating the smooth muscle, but appear mediated from specialised pacemaker cells (eg: interstitial cells), distinct from the smooth muscle cells themselves (Takaki et al., 2010).

Spontaneous contractions in the bladder

Urodynamically, the whole bladder develops spontaneous non-voiding contractions during filling. Tonic contractions occur in the urethra while phasic contractions occur in the detrusor (Fry et al., 2010, Brading, 2006). Traditionally, this activity was thought to be associated with abnormal bladder function, however, it is now understood that spontaneous contractions during filling are evident in healthy human bladders (van Waalwijk van Doorn et al., 1992, Robertson, 1999).

However, the importance for the size of each bladder spontaneous contraction is uncertain and large amplitude spontaneous bladder activity may have a role in evoking afferent mechanisms; initiating sensations of bladder fullness or pain; maintaining muscular tone; or triggering voiding and urine storage reflexes (Fry et al., 2010, Brading, 2006).

The normal role of spontaneous bladder contractions is unclear, however, they have been shown to change in frequency and magnitude during various disorders of the lower urinary tract. Patients suffering from overactive bladder may exhibit larger amplitude, higher frequency spontaneous contractions in isolated detrusor tissues *in vitro* (Fry et al., 2004b), or exhibit a higher bladder contractile index than healthy patients (Oh et al., 2011). These increases in spontaneous activity during the filling stage are common identifying features used during urodynamic observations to diagnose patients with detrusor overactivity (Rovner and Goudelocke, 2010, Fan et al., 2011).

Bladder outlet obstruction in mice causes the spontaneous contractions to decrease in frequency but increase in magnitude, simulating the symptoms of overactive bladder (Drake et al., 2003d). Damage to the nervous system is also known to result in overactive bladder (Fowler et al., 2008), and this can be replicated in animal models through transection of the spinal cord, which increases the amplitude and frequency of spontaneous contractions in rat bladders (Ikeda et al., 2007). The origins of the increases in spontaneous contractile activity during pathology are not known. However, they may be related to changes in the properties of the smooth muscle; the inability of the detrusor muscle to correctly regulate intracellular calcium (Fry et al., 2002); increases in electrical conductivity with alterations of gap junctions (Wang et al., 2001, Haferkamp et al., 2003); changes in receptor or ion channel function (Turner and Brading, 1997); abnormal levels of muscle oxygenation (Vijaya et al., 2012); chemical modulator release from the urothelium (Birder and de Groat, 2007); or an increased distribution of interstitial cells (Kubota et al., 2008).

Altering the frequency of the spontaneous contractile activity may be an action of antimuscarinic drugs prescribed to alleviate the symptoms of overactive bladder, although their precise mechanisms and sites of action have yet to be established (Sellers and Chess-Williams, 2012). Nonetheless, such drugs are the preferred pharmacological treatment for urgency, frequency, and incontinence associated with bladder overactivity (Andersson, 2004a, Chapple, 2000, Kumar et al., 2003, Nijman, 2004, Rovner and Wein, 2002, Yono et al., 2000).

Detrusor spontaneous activity

The spontaneous contractions of the bladder appear to arise from contractile activity within the detrusor smooth muscle. This suggests that an increased rate or amplitude of detrusor contractions may result in an enhanced urge to urinate and thus be an underlying cause of overactive bladder (Coolsaet et al., 1993, Al-Ghazo et al., 2011, Kinder and Mundy, 1987, Mills et al., 2000). The origin of these spontaneous contractions is not certain. They do not arise from neuronal sources (Drake et al., 2003a), but are generated from within the detrusor smooth muscle itself (Drake et al., 2003b, Lagou et al., 2006), and possibly mediated by interstitial cells, identified within the detrusor (Davidson and McCloskey, 2005). This spontaneous activity can be modulated through increased extracellular magnesium ions or by calcium agonists (Montgomery et al., 1992, Levin et al., 1991). In addition, a range of other agonists, many of which are released endogenously from the urothelium/lamina propria, modulate detrusor activity. For example, in animal models the contractions can be increased through exogenous ATP; nitric oxide; and acetylcholine. Conversely, the contractions can be inhibited by noradrenaline; calcitonin gene-related peptide; and via the inhibition of phosphodiesterases (Gillespie et al., 2003, Drake et al., 2003d, Finney et al., 2008, Finney et

al., 2007, Streng et al., 2006, Gillespie, 2004c, Gillespie, 2004b). It is still uncertain as to what extent the findings in animal models relate to human bladders. Research in humans has measured only large contractions of the bladder through cystometry, whereas animal whole-bladder models have been used to measure a wider range of pressure increases. This method in animal bladders has identified a number of different variables in the spontaneous activity (Biallosterski et al., 2011, Streng et al., 2006). As such, it is unknown whether isolated whole bladder preparations in humans have a similar range of contractile pressures during filling or if they respond in similar ways to receptor activation.

1.4.1 Role of the urothelium and lamina propria in regulating detrusor spontaneous contractile activity

As the bladder fills the urothelium/lamina propria is stretched and the cells within this layer change their shape to accommodate an increasing volume. To accomplish this, sensors on the urothelium respond to stretch by releasing chemical transmitters. These allow the bladder wall to relax, and thus facilitate increases in volume; or activate the sensory innervation to the area (Figure 1-10). Upon stretching, acetylcholine is released endogenously from cells within the urothelium (Yoshida et al., 2006, Yoshida et al., 2004b, Hanna-Mitchell et al., 2007), along with ATP (Young et al., 2012, Ferguson et al., 1997, Cheng et al., 2011b). Other chemicals released during stretch of the urothelium include nitric oxide which results in tissue relaxation (Ferguson et al., 1997, Birder et al., 1998), and prostaglandin E2 (Yoshida et al., 2004a) which can stimulate bladder contractions (de Jongh et al., 2007b, Kobayter et al., 2012).

Many of the chemicals released from the urothelium and lamina propria during the filling stage may modulate the overall bladder spontaneous contractile activity. One main regulator of bladder spontaneous contractions in rats appears to be acetylcholine, which may enhance spontaneous activity in the intact bladder (Kanai et al., 2007). Although acetylcholine is released by nerves in the detrusor, usually resulting in muscle contraction, it can also be released from the urothelium which shows positive immunohistochemical staining for choline acetyltransferase (Yoshida et al., 2008). This urothelial-derived acetylcholine could act directly on the detrusor, or on the interstitial cells in the lamina propria, potentially influencing the spontaneous activity of the bladder (Gillespie et al., 2003, Fry et al., 2007). Furthermore, in experiments on human bladder strips this non-neuronal release of acetylcholine was increased in both aged rats and bladder stretching (Yoshida et al., 2008). There is also evidence to support the existence of purinergic receptors in the urothelium/lamina propria although their exact functional role is unclear. ATP released from the urothelium/lamina propria in mice is thought to activate the afferent nerves adjacent to the urothelium (Rong et al., 2002). This is via the P2X-purinergic receptors on both A δ - and C-fibre afferent nerves in the lamina propria (Kumar et al., 2004, Munoz et al., 2010, Yoshimura et al., 2008a, Du et al., 2007). Additionally, in rats ATP also stimulates P2Y-purinergic receptors on interstitial cells in the lamina propria which increases the frequency of calcium transient activity through the tissue (Fry et al., 2012).

In addition to releasing chemical transmitters to directly influence detrusor contractile activity, another role for the urothelium/lamina propria may be the generation and modulation of bladder spontaneous activity (Kanai et al., 2007, Fry et al., 2012). It has been identified that coordinated spontaneous activity in the rat bladder requires an up-regulation of gap junctions between cells within the urothelium and lamina propria (Ikeda et al., 2007).

Through using an optical mapping technique which traces the flow of charge through rat bladders, it was observed that the spontaneous activity, at least in regards to the calcium transients through the tissue, was generated from within the urothelium and modulated by structures within the lamina propria (Ikeda and Kanai, 2008). A later study supported this in rat tissue, observing that calcium transients are initially generated in the lamina propria and then spread outwards to both the urothelium and detrusor (Fry et al., 2012). Although the link between spontaneous calcium transients and the spontaneous contractile activity has not been established in many species, these finding still indicate the importance of the urothelium/lamina propria in generating, coordinating and modulating spontaneous activity within the detrusor. Functional studies have also shown that the presence of the urothelium/lamina propria is associated with an increased bladder spontaneous activity (Sui et al., 2008, Akino et al., 2008). Alternatively, a urothelial derived inhibitory factor (UDIF) is thought to be released from the urothelium/lamina propria and appears to depress responses to carbachol and histamine (Templeman et al., 2002a). Although the relationship between the urothelium/lamina propria and bladder spontaneous contractions is still uncertain, these recent studies have identified a clearly important role for the tissue, and the potential for a malfunctioning urothelium/lamina propria to be an underlying cause or contributor in bladder diseases.

1.4.2 Spontaneous activity of the urothelium/lamina propria

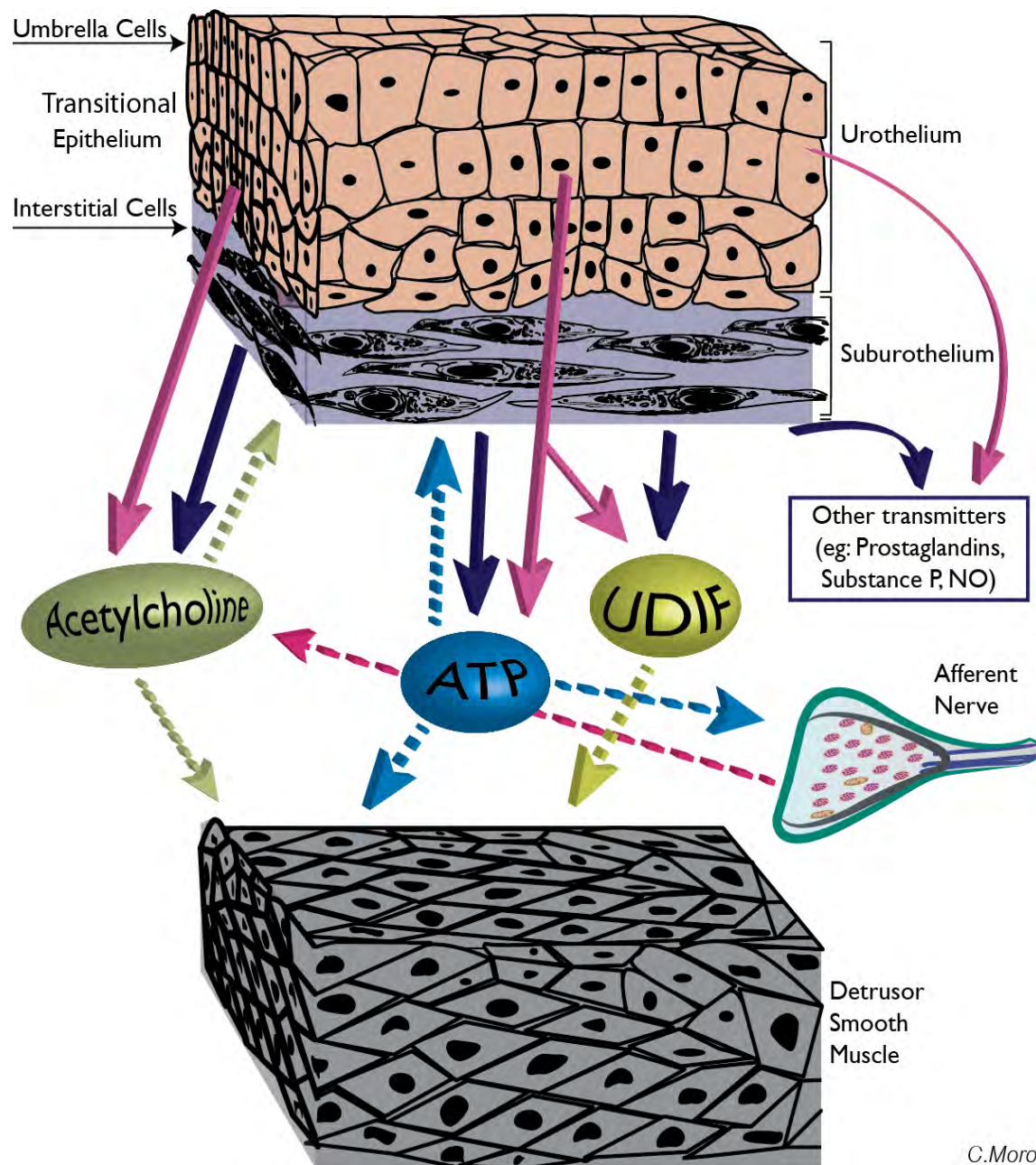
The urothelium/lamina propria has an ability to release various modulators of sensory neurons and smooth muscle which can influence detrusor spontaneous contractions (Birder and de Groat, 2007, Kumar et al., 2005, Buckner et al., 2002, Hawthorn et al., 2000, Kanai et al., 2007). However, the isolated urothelium/lamina propria is capable of generating its own

spontaneous contractions (Sadananda et al., 2008) and these contractions may stimulate afferent nerves in the lamina propria; maintain bladder tone; assist the urothelium to fold into its rugae; or directly influence the detrusor to maintain normal continence during the filling stage.

It is important to identify the possible cell types that may be responsible for the generation, propagation and pacemaking of the lamina propria contractile activity. Interstitial cells are usually presented as the likely regulators of bladder spontaneous activity (Fry et al., 2004b, Montgomery and Fry, 1992). Although the exact role for interstitial cells is unclear, they could facilitate the spontaneous activity by modulating the contractile amplitude and acting as pacemakers to regulate the frequency (Wiseman et al., 2003, Fry et al., 2004a, Gillespie et al., 2006b). This role for lamina propria interstitial cells would also relate to their morphological similarities with the ICC cells in the gut, which are responsible as pacemaking cells regulating contractions.

The contractile activity of the urothelium/lamina propria may also be generated by muscle cells. These are located between the basement layer of the urothelium and the muscularis of the detrusor, and termed the muscularis mucosae. These muscle cells have been extensively studied, although usually in relation to their potential role in carcinomas of the bladder (Chaimuangraj et al., 2006, Paner et al., 2007, Weaver and Abdul-Karim, 1990). There is a hypothesis that the muscularis mucosae is involved in the regulation of spontaneous contractile activity in the urothelium, and that the ability to contract with a cyclical frequency demonstrates its potential to work as a pacemaker or regulator (Heppner et al., 2011). However, this hypothesis is not widely accepted and in comparison to the detrusor, it is thought that sparse smooth muscle exists in the urothelium/lamina propria (Cheng et al.,

2011b). Furthermore, removal of the smooth muscle cells from the urothelium/lamina propria does not appear to affect contractile responses (Sadananda et al., 2008). Smooth muscle actin is also present in the lamina propria interstitial cells which suggests that the observed spontaneous contractile activity may arise from these interstitial cells rather than a specialised muscle layer (Drake et al., 2006, McCloskey, 2010, Gevaert et al., 2011, Cheng et al., 2011b).



C.Moro

Figure 1-10: Urothelial-derived transmitters acting on the detrusor smooth muscle and afferent innervation. During the filling stage, transmitters are not only released by nerves within the bladder, but can be released from the urothelium and lamina propria. Increased stretch and pressure leads to acetylcholine release from both nerves and the urothelium. ATP, urothelial derived inhibitory factor (UDIF), prostanoids, substance P, nitric oxide and nerve growth factor are also known to be released and may act on the lamina propria and afferent nerves. Image collated from a range of references including: (Fry et al., 2010, Birder and de Groat, 2007, Hanna-Mitchell et al., 2007, Apodaca, 2004, Yoshida et al., 2006, Yoshida et al., 2004a, Ferguson et al., 1997).

1.5 RECEPTORS AND SYSTEMS IN THE URINARY BLADDER

1.5.1 Cholinergic receptors

1.5.1.1 *Muscarinic receptors*

Muscarinic receptors are the main receptors mediating detrusor muscle contraction and as such, they are an important target in the pharmacological management of bladder diseases (Andersson, 2011, Yamaguchi, 2010). Activation of the postjunctional muscarinic receptors was previously thought to be mediated exclusively by acetylcholine released from cholinergic nerves, which run throughout the detrusor muscle and into the lamina propria (Dixon et al., 2000). However, it is now understood that acetylcholine is also released from the urothelium, identifying a non-neuronal mechanism of receptor activation (Yoshida et al., 2006, Yoshida et al., 2008, de Groat, 2004).

The muscarinics are 7-transmembrane receptors comprised of 5 subtypes, which have been classified as $M_1 - M_5$ via both pharmacological and molecular characterisation (Caulfield and Birdsall, 1998, van Koppen and Kaiser, 2003). Of these, M_1 , M_2 and M_3 have been well characterised pharmacologically, primarily due to the availability of relatively selective antagonists. Although there are similarities in their structure, the signal transduction system varies between the subtypes (Giglio and Tobin, 2009). All five muscarinic receptors couple to G-proteins, with M_1 , M_3 and M_5 coupling to $G_{q/11}$ (Figure 1-11). This leads to increased intracellular calcium release from the sarcoplasmic reticulum (SR) via inositol 1,4,5-triphosphate (IP3), resulting in smooth muscle contraction (Wu et al., 2000). In addition to the SR release, calcium also enters the cell through the membrane via the diacylglycerol (DAG)-mediated activation of protein kinase C and opening of L-type calcium channels

(Berridge, 1984, Streb et al., 1983, Nishizuka, 1984, Schneider et al., 2004). It is generally thought that the M₂ and M₄ muscarinic receptor subtypes act in an inhibitory role in bladder function (Andersson, 2011). These receptors couple to G_{i/o} and affect the activity of adenylate cyclase (Hegde et al., 1997, Londos et al., 1981); inhibit potassium channels (Bonev and Nelson, 1993, Nakamura et al., 2002); and inhibit nonselective cation channels (Kotlikoff et al., 1999, Yamamoto et al., 2008). The importance of the muscarinic receptors in the management of bladder function is highlighted by the fact that the main class of drugs used to treat overactive bladder are clinical muscarinic antagonists. These include solifenacin, oxybutynin, tolterodine, darifenacin and trospium (Athanasopoulos and Giannitsas, 2011). The mechanisms of action for these drugs is not fully understood; however, they likely target the muscarinic receptors expressed throughout the lower urinary tract and bladder (Hegde, 2006).

Muscarinic receptors in the ureters

The function of muscarinic receptors in the ureter is not clear. Ureters from patients undergoing radical nephrectomy were examined in a study which observed mRNA expression for only the M₂, M₃ and M₅ receptors, although the presence of all subtypes was demonstrated through immunohistochemistry (Sakamoto et al., 2006). However, this study did not contain an adequate sample size (only 3 for RT-PCR and 4 for immunohistochemical analysis), and there was the confounding factor of the patient's kidney diseases, which may affect the ureters. In addition, this study heavily relied upon immunohistochemistry for the conclusion, yet did not present adequate controls to address the problems associated with the lack of specificity in current muscarinic and G-protein coupled receptor antibodies (Pradidarcheep et al., 2008, Pradidarcheep et al., 2009, Jositsch et al., 2009, Michel et al.,

2009). The functional role of the muscarinic receptors in the ureter is still uncertain, although they are suggested to be involved in stimulation of muscle contraction in human, canine, equine and porcine models (Canda et al., 2007, Long and Nergardh, 1978, Tomiyama et al., 2004, Hernandez et al., 1993, Prieto et al., 1994).

Muscarinic receptors in the urethra

The urethra, where sympathetic nerves have the predominant role functionally, has also been found to receive a dense cholinergic innervation in both human and animal models (Ek et al., 1977a, Persson et al., 1995, Arrighi et al., 2008). The role of this neuronally released acetylcholine in the urethra is uncertain (Andersson, 2011) although it may act on M₁, M₂ or M₃-muscarinic receptor subtypes, as identified through functional studies and radioligand binding in rabbit urethra (Mutoh et al., 1997, Nagahama et al., 1998). Nonetheless, predominant research into receptor expression in the human urethra has focussed on the adrenoceptor system and there remains a paucity of research analysing the muscarinic receptor population and function.

Muscarinic receptors in the prostate

The exact function and expression of individual muscarinic receptor subtypes in the prostate is uncertain. Molecular biological studies have demonstrated mRNA expression in human prostate cells for all muscarinic receptors (M₁ - M₅), with a strong expression of M₂ in the stromal cells compared to the other receptors (Obara et al., 2000). This was consistent with previous studies using radioligand binding (Yazawa et al., 1994). Obara (2000) also concluded the expression of M₁, M₂, and M₅-muscarinic receptors in prostatic epithelial cell

cultures, although the results and images presented in the paper did not display clear M₁-transcripts, with questions arising as to the validity of the conclusion. Further radioligand binding assays in human and rat prostate has determined that only the M₁ and M₂-muscarinic receptor subtypes are present (Anisuzzaman et al., 2008), although this study relied on only one M₃-selective antagonist, darifenacin, for this conclusion. Functionally, recent research using electrical field stimulation and exogenously applied cholinergic agonists has identified the M₃ muscarinic receptor as the subtype mediating the cholinergic component of contraction in mouse prostate (White et al., 2011). It will be of interest to examine further roles for these receptors in the contractile function of human prostate, which may lead to additional targets for the pharmacological treatment of benign prostatic hyperplasia.

Muscarinic receptors in the bladder

Human detrusor muscle expresses all five muscarinic receptor subtypes at the mRNA level (Sigala et al., 2002), with a greater expression of the M₂-muscarinic receptors relative to the M₃-muscarinic receptors (Homma and Yamaguchi, 2009, Yamaguchi et al., 1996, Giglio and Tobin, 2009). This was similar to that found in pig bladder (Maeda et al., 1988). Rat urinary bladder also has a predominant expression of the M₂ and M₃ subtypes, and although these bladders were thought to express only the M₁ – M₄ muscarinic subtypes (Braverman et al., 1998), recent studies have identified a low expression of the M₅-receptor (Creed et al., 2010). At the protein level, human bladder contains ~70% M₂ receptors, ~20% M₃-receptors and ~10% M₁-receptors, as detected by competition radioligand binding (Mansfield et al., 2005) and immunoprecipitation (Wang et al., 1995, Goepel et al., 1998). A higher proportion of the M₂-muscarinic receptor subtype (~90%) is also seen in mice (Choppin and Eglen, 2001b),

rats (Choppin et al., 1998, Hegde et al., 1997, Longhurst et al., 1995, Longhurst and Levendusky, 2000, Tong et al., 1997), rabbit and guinea pig (Wang et al., 1995).

Although the M₂-subtype is the most prevalent in the bladder, it is the M₃-subtype that is responsible for contraction in all species studied to date. During voiding, acetylcholine is released from the neuroeffector junctions of both ganglionic and postganglionic nerves (Persson et al., 1995, Smet et al., 1996a) where it then acts predominantly on receptors in the detrusor smooth muscle. It has been ascertained that the M₃-muscarinic receptor subtype is responsible for direct contraction in human (Chess-Williams et al., 2001, Fetscher et al., 2002), porcine (Sellers et al., 2000b), dog (Choppin and Eglen, 2001a), mouse (Choppin and Eglen, 2001b) and rabbit (Tobin, 1995) bladders. This is further supported by mice lacking the M₃-receptor, through gene knockout, demonstrating a greatly reduced detrusor contractility (Matsui et al., 2002). In addition, M₃-receptor knock-out mice exhibit bladder distension and develop urinary retention, yet this does not occur in M₂ knock-out mice (Matsui et al., 2000). The mechanisms underlying this M₃-receptor mediated contractile response in the detrusor are outlined in Figure 1-11. The role of the M₂-muscarinic receptors in the bladder appears slightly more complex. There is a small effect on cystometric parameters in mice lacking the M₂-receptor via gene knockout (Igawa et al., 2004, Ehlert et al., 2005) and it is understood that the M₂-receptors may oppose the sympathetically-mediated detrusor relaxation through inhibition of adenylyl cyclase (Hegde et al., 1997). Alternatively, M₂-receptors may have a function during periods of high sympathetic activity, or play a greater role when the M₃-receptors are dysfunctional (Yamanishi et al., 2001). Nonetheless, the functional role of the M₂-muscarinic receptor subtype in the bladder is still not clearly understood.

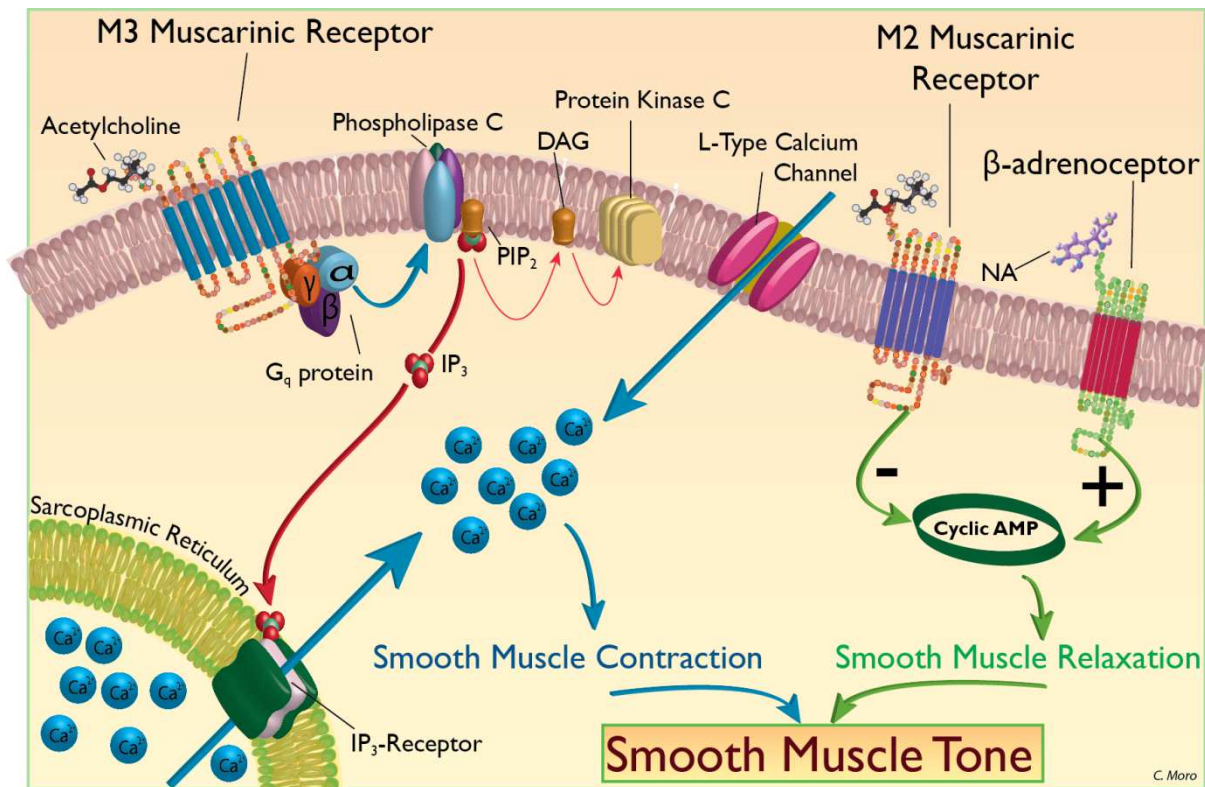


Figure 1-11: Diagram representing the intracellular actions of acetylcholine acting on M₂ and M₃ muscarinic receptor subtypes. The M₃-receptor acts via the second messengers inositol triphosphate (IP₃) and diacylglycerol (DAG) to result in smooth muscle contraction. Additionally, M₂-receptors may inhibit cyclic AMP and reduce contraction. In conjunction with the β-adrenoceptor mediated increase in cyclic AMP, may maintain smooth muscle tone.

Muscarinic receptors in the urothelium/lamina propria

In the urothelium/lamina propria of human bladders all muscarinic receptors are expressed at the mRNA level, with a high expression of the M₂ and M₃ subtypes (Mansfield et al., 2005, Bschleipfer et al., 2007, Ochodnický et al., 2012). Immunohistochemical studies have also identified a high prevalence of the M₂ and M₃ receptors in human urothelium/lamina propria (Mukerji et al., 2006b, Tyagi et al., 2006). An interesting finding in porcine bladders, was that the density of muscarinic receptors was found to be higher in the urothelium/lamina propria than in the detrusor (Hawthorn et al., 2000), which is in direct contrast to rat tissue where the density is higher in the detrusor (Anisuzzaman et al., 2008). However, these

differences may be due to experimental variations in exposure of the tissues to the ^3H -QNB and carbachol. Anisuzzaman *et al* (2008) found that receptor density, as measured using ^3H -QNB, was dependent upon the concentration and the time of incubation.

The cell type distribution of the muscarinic subtypes in the urothelium and lamina propria is still uncertain. Within human bladders the M_1 receptors appear expressed exclusively on the urothelial basal cells, while the M_2 -receptor is most dense in the umbrella cell layer (Bschleipfer *et al.*, 2007). An immunohistochemical study has also identified both M_2 and M_3 -receptors on nearby nerve fibres as well as the urothelium (Mukerji *et al.*, 2006b). An exciting prospect is the suggestion that the M_3 -muscarinic receptor subtype is located on the interstitial cells in the lamina propria, as demonstrated by immunohistochemical staining in the guinea pig (Grol *et al.*, 2009). These muscarinic receptors may present a potential mediator of bladder activity, and may influence the activity of the interstitial cells. The urothelium and possibly the lamina propria can release non-neuronal acetylcholine, which may be a mechanism whereby the urothelium modulates detrusor contraction during filling or voiding via muscarinic receptors (Lips *et al.*, 2007, Yoshida *et al.*, 2006, Hanna-Mitchell *et al.*, 2007, Kawashima and Fujii, 2008). The urothelium and the lamina propria also responds to muscarinic agonists, demonstrating dose-dependent increases in intracellular calcium (Gupta *et al.*, 2009). Therefore, the expression and function of these muscarinic receptors on this tissue layer is important for the overall understanding of bladder activity.

Presynaptic muscarinic receptors

Prejunctionally, the M_1 -muscarinic receptor subtype appears to facilitate the release of acetylcholine in human (Somogyi and de Groat, 1999) and rat urinary bladders (Braverman *et*

al., 1998, Somogyi and de Groat, 1992). There is also a role for the M₄ receptor subtype as a prejunctional receptor which inhibits acetylcholine release in the bladders of rabbit (Tobin and Sjogren, 1995, Inadome et al., 1998), guinea pig (Alberts, 1995) and mouse (Takeuchi et al., 2008), and the prejunctional inhibitory muscarinic receptor also appears to be M₄-subtype in humans (D'Agostino et al., 2000). In the rat bladder the inhibitory prejunctional muscarinic receptor subtype has been argued to be the M₄-receptor (D'Agostino et al., 1997, Shen and Mitchelson, 2001), or conversely the M₂-muscarinic receptor subtype (Somogyi and de Groat, 1992, Braverman et al., 1998). In studies by Somogyi and de Groat (1992) and Braverman *et al* (1998), the conclusions of M₂-mediated inhibitory effects were highly reliant upon the relatively selective M₂-antagonist methoctramine, and the M₁-selective antagonist pirenzepine at a limited range of concentrations. Both D'Agostino et al (1997) and Shen *et al* (2001) used a wider range of muscarinic receptor antagonists and concentrations to determine that the inhibition is mediated by the M₄-muscarinic receptor subtype. Although full concentration-response curves were not completed, and thus pA₂ values not calculated, the latter conclusion, implicating the M₄-receptor, appears to be better proven experimentally.

The lack of highly selective antagonists for the M₄ or M₅ receptor subtypes has limited this research. Studies using gene knock-mice show that the M₄-receptor appears to be a promising prejunctional receptor as a regulator of normal bladder activity. In M₂-knockout mice, bladders that were pre-incubated with [³H]-choline and a muscarinic antagonist, could induce a tritium outflow signifying the release of acetylcholine, yet this did not occur in M₄ knock-out mice (Zhou et al., 2002). These researchers suggested that the autoreceptors in the mouse bladders are exclusively M₄. However, a prior study had ruled out using the [³H]-choline as it does not tend to label all of the released acetylcholine (Shen and Mitchelson, 2001). A later study using M₄ knock-out mice confirmed that prejunctional inhibitory

muscarinic receptors were most likely the M₄-receptor subtype (Takeuchi et al., 2008). Nonetheless, due to contradictory results and methods, it is clear that further controls need to be conducted in these knock-out studies prior to a clear confirmation of an M₄-mediated effect, although the potential for these subtypes to induce functional effects does offer an exciting avenue of future research.

Clinical antimuscarinics in bladder disease

Antimuscarinics are the most common pharmacological treatment for bladder diseases such as overactive bladder. The exact mechanisms by which these antimuscarinics alleviate the symptoms of pathologies such as overactive bladder are still uncertain. The currently accepted hypothesis is that they act on muscarinic receptors in the detrusor, blocking the M₃-receptors and reducing the detrusor's ability to contract (Abrams et al., 2006). However, in relation to studies of muscarinic receptor antagonists in animal models, comparatively small doses of antimuscarinics are used in human patients, and at these doses they do not seem to significantly influence voiding contractions (Andersson, 2004a, Abrams et al., 2006). Therefore, it seems that the effect of these drugs is solely during the filling/storage phase, thus likely antagonising the acetylcholine released from the urothelium (Andersson and Yoshida, 2003). They may also assist in minimising the responses due to an increased density of urothelial/lamina propria M₂ and M₃ muscarinic receptors, known to be associated with overactive bladder in humans (Mukerji et al., 2006b) and in models of bladder outlet obstruction in rats (Kim et al., 2008). Based on results from animal studies, reviews have also suggested that the antimuscarinics act upon the urothelial afferent nerve activity (Yamaguchi, 2010, Finney et al., 2006). This has been shown indirectly through the measurements of voiding intervals in rats which were administered muscarinic agonists and

antagonists (Kullmann et al., 2008, Finney et al., 2006), and also from directly measuring nerve afferent activity (Yu and de Groat, 2010).

There is a further hypothesis that an increased release of acetylcholine might be responsible for the symptoms of bladder overactivity (Yoshida et al., 2008). However, in mice, acetylcholine acting on muscarinic receptors appeared to depress sensory transduction (Daly et al., 2010a). This is a controversial proposal in contrast to past studies which have suggested that increases in acetylcholine release contributes to bladder diseases such as overactive bladder through increasing afferent nerve activity (Yoshida et al., 2008). Subsequent discussions related to these findings have demonstrated a scientific interest in the bladder afferent function and the responses to acetylcholine (Daly et al., 2010b, Behr-Roussel and Giuliano, 2010).

In overactive bladder and animal models of bladder dysfunction, the density of muscarinic receptors and detrusor sensitivity to acetylcholine is increased (Mukerji et al., 2006b, Stevens et al., 2007, Stevens et al., 2006). This finding is not consistent across all models, and rats with benign prostatic hypertrophy, which is known to induce overactive bladder, have demonstrated a *hyposensitivity* to muscarinic agonists without signs of denervation (Lluel et al., 2002). Studies have also attempted to associate the muscarinic receptors with interstitial cystitis (IC), providing a potential for antimuscarinics to be trialled as a treatment. Bladder urothelial cells from patients suffering IC were found to have an increased sensitivity to the muscarinic agonist carbachol (Gupta et al., 2009). However, IC is such a broad and undetermined pathology and the fact that only 4 patient samples were examined, means that this conclusion may not be representative. Additionally, the increased sensitivity demonstrated in this study was only evident at 10 μ M – 1 mM concentrations of carbachol,

which is not necessarily physiologically relevant. Nonetheless, it is important to identify therapeutic targets in the bladder that may alleviate the symptoms of overactive bladder or interstitial cystitis. The current use of antimuscarinics is the most common treatment for overactive bladder yet there has been no randomised placebo-controlled study identifying the effectiveness of muscarinic antagonists in alleviating the symptoms of interstitial cystitis.

1.5.1.2 *Nicotinic receptors in the bladder*

The bladder expresses mRNA for the α_1 - α_7 , β_1 - β_4 nicotinic subunits, as identified using RT-PCR in rat urothelial cells. However, only α_3 - (excitatory) and α_7 - (inhibitory) subunits are translated into proteins, as detected by subsequent western blotting (Beckel et al., 2006, Yamamoto et al., 2011). It has been suggested that there is an inhibitory nicotinic receptor on cholinergic nerve terminals of the rat urinary bladder (Somogyi and de Groat, 1992), although the physiological relevance of this is yet to be investigated.

The possible role of nicotinic receptors in lower urinary tract dysfunction is not clearly understood. The main response to acetylcholine in the bladder is mediated via cholinergic muscarinic receptors. Therefore, it is not commonly thought that neuronally released acetylcholine acts upon nicotinic receptors to induce functional effects within the bladder. However, functional roles suggested for these urothelial nicotinic receptors may include stimulating calcium or ATP release from the tissue (Beckel and Birder, 2012).

Application of intravesical nicotine affected bladder reflexes in rats *in vivo*, as well as increasing intracellular calcium concentrations in cultured urothelial cells (Beckel et al., 2006). The authors of that study were able to block the nicotinic response in cultured cells

with the antagonist hexamethonium, yet when applied intravesically this drug had no effect. This may either suggest that nicotine has a greater effect on cultured cells than on normal bladder function, or that nicotine's effect is not localised to the urothelial layer but acts on other areas in the bladder. In addition, other nicotinic agonists showed inconsistent results, such as epibatidine, which gave initial excitatory responses, then subsequently caused urinary retention (Beckel et al., 2006). DMPP, a nicotinic receptor agonist, can stimulate postganglionic action potentials in the rat bladder and cause the release of nonadrenergic noncholinergic neurotransmitters which stimulate contraction (Tong et al., 1996) although the released transmitters and targeted receptors are still unclear.

There is the potential that therapeutic agents used to treat overactive bladder, such as distigmine, may also have an effect via its actions on the nicotinic receptors (Harada et al., 2010). These authors observed a clear distigmine binding to nicotinic receptors in the rat cerebral cortex, although the alterations in bladder activity appeared to be due solely to its effects on the muscarinic receptors. A further hypothesis stems from the suggestion that activation of the nicotinic pathway is involved in the down regulation of inflammatory cytokine production in a number of human diseases including respiratory infections and inflammatory diseases (Ulloa and Wang, 2007, Lakhan and Kirchgessner, 2011). This process may also have implications for bladder diseases, and researchers have identified that activation of nicotinic receptors leads to an attenuation of inflammatory mediator production in the bladder (Martinez-Ferrer et al., 2008, Starkman et al., 2008). This conclusion was arrived at after histological (H&E staining), immunohistochemical and gene expression analyses of rat bladders that underwent chemical-induced inflammation. These results identify the nicotinic receptors as potential therapeutic targets for bladder diseases, in

particular, those commonly associated with chronic inflammation and further research into these mechanisms is required.

1.5.2 Adrenoceptors

The adrenoceptors are activated by noradrenaline released from sympathetic nerves innervating the bladder (Figure 1-12). Throughout the body, α -adrenoceptors and the β -adrenoceptors mediate a variety of functions. Generally, α -adrenoceptors play excitatory roles and can be divided into two characterised receptor subtypes, the α_1 (consisting of α_{1A} , α_{1B} and α_{1D} with a possible α_{1L} conformation) and α_2 -adrenoceptors. In contrast, activation of β -adrenoceptors tends to lead to inhibitory responses, and these receptors have been classified into the subtypes β_1 , β_2 and β_3 (Alexander et al., 2011).

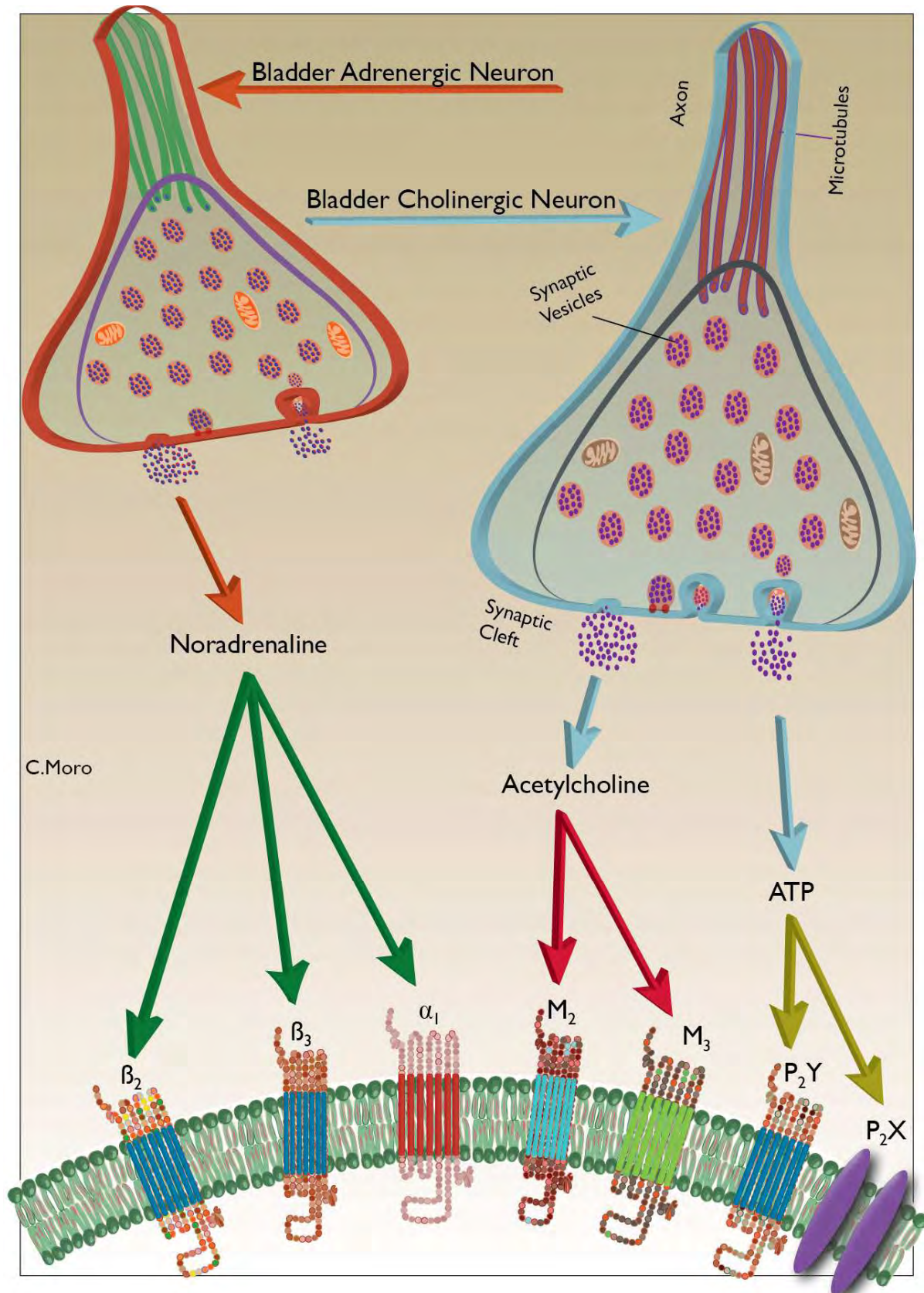


Figure 1-12: Illustration of common neurotransmitters released from the bladder. Noradrenaline, acetylcholine and ATP are released from adrenergic and cholinergic nerves, and act on the nearby expressed and functionally active receptors.

1.5.2.1 *β-adrenoceptors*

β-adrenoceptors in the lower urinary tract

It is understood that the β-adrenoceptors have a clear role throughout the lower urinary tract, yet their distributions vary. In the human ureters, although the mRNA for all three β-adrenoceptor subtypes is expressed, only relatively low concentrations of β-adrenoceptor protein has been identified with radioligand binding (Park et al., 2000). Functionally, the β-adrenoceptors in the ureters appear to have no role, with noradrenaline-induced contraction mediated via the α₁-adrenoceptors. However, there is evidence for a relaxation response to noradrenaline in the distal ends of the ureters, potentially mediated via β-adrenoceptors (Tindall, 1972). In the prostate, β-adrenoceptors may have a role in inhibition of noradrenaline-invoked α₁-adrenoceptor mediated contractions in animals and humans (Kalodimos and Ventura, 2001, Haynes and Hill, 1997, Normandin and Lodge, 1996, Garcia-Sacristan et al., 1984, Tsujii et al., 1992, Drescher et al., 1994). The presence of β-adrenoceptors is also noted in the urethra, where there is evidence for a β-adrenoceptor mediated relaxation in humans (Thind et al., 1993), although the α-adrenoceptor contractile response also predominates in this tissue.

β-adrenoceptors in the bladder

All three β-adrenoceptor subtypes couple via G-proteins to adenylyl cyclase. This leads to an increase in the levels of intracellular adenosine 3':5'-cyclic adenosine monophosphate (cAMP) which subsequently activates protein kinase A. During bladder filling there is no large elevation of intravesical pressure within the bladder lumen. In order to accommodate

the increasing volume, the detrusor relaxes (Andersson and Arner, 2004), and it is thought that this may, at least partly, be facilitated via β -adrenoceptors (Michel and Sand, 2009). Activation of β_3 -adrenoceptors causes the opening of large-conductance calcium-activated potassium channels in the urinary bladder smooth muscle, resulting in membrane hyperpolarisation and relaxation (Hristov et al., 2008). β -adrenoceptors may also assist in reducing smaller spontaneous contractions which may allow a more stable detrusor during filling (Klausner et al., 2009), and a constant stimulation of these receptors would potentially alleviate the bladder from becoming overactive during pathological circumstances.

The β -adrenoceptors in the bladder mediate responses to noradrenaline released from the sympathetic nervous system (Andersson and Arner, 2004). For many years these β -adrenoceptor responses were thought to arise specifically from the β_1 - and β_2 -receptor subtypes, although since then a third subtype has been identified and termed the β_3 -adrenoceptor (Alexander et al., 2011, Bylund et al., 1994). These three currently identified subtypes have been cloned and classified, yet much less appears to be known about the role of the β_3 -receptor in particular. This is due to the range of pharmacological tools available to selectively target the β_1 - and β_2 -adrenoceptor subtypes, whereas the more recently developed agonists and antagonists that target the β_3 -receptor subtype have not yet been proven in a wide range of experimental studies. However, some antagonists have shown promise in selectively targeting the β_3 -adrenoceptor, such as SR59230A (Manara et al., 1996) and L748337 (Candelore et al., 1999). Alternatively, available agonists such as CL316,243 are claimed to be highly selective at the β_3 -adrenoceptors in some animals models (Clouse et al., 2007, Bloom et al., 1992). Although these are some of the current pharmacological tools for understanding the responses of the β_3 -adrenoceptor, there is controversy surrounding their selectivity. For example, novel β_3 -antagonists such as SR59230A may lack selectivity in

humans, or even have a partial agonist affect accompanying the β_3 -antagonism (Sato et al., 2007, Michel and Vrydag, 2006).

At the molecular level it has been found that β_3 -adrenoceptors account for 97% of the mRNA expression in the human bladder, with 1.5% and 1.4% for the β_1 and β_2 respectively (Nomiya and Yamaguchi, 2003). However, it is unwise to draw functional conclusions from this data, since β -adrenoceptor subtypes detected using reverse-transcription-PCR do not always correspond to those which mediate the smooth muscle relaxation. In support of these findings, however, immunohistochemical studies have also detected the expression of all three β -adrenoceptor subtypes in the human urinary bladder (Otsuka et al., 2008). Pig and human detrusor smooth muscle relaxation may be due to activation of both β_2 and β_3 with the latter predominating as the main functional receptor (Yamanishi et al., 2002b, Igawa et al., 2001, Takeda et al., 1999).

The distribution of β -adrenoceptors in the bladder and the corresponding β -adrenoceptor subtype responsible for bladder relaxation is still under investigation in many species. Studies utilising radioligand binding in the urinary bladder have reported the highest densities of the β_2 -subtype in pigs (Goepel et al., 1997) and rabbits (Latifpour et al., 1990). However research into the range of radioligands used in these studies concluded that they have significantly lower affinities than previously thought, suggesting that more effective ligands and further studies utilising high-affinity β_3 -radioligands must be performed (Niclauss et al., 2006, Hoffmann et al., 2004). In addition, future research into the localisation of these subtypes may prove difficult as antibodies used to discriminate against the three β -adrenoceptor subtypes appear to lack selectivity and their use has been criticised (Hamdani and van der Velden, 2009, Pradidarcheep et al., 2009). This criticism is due to the low

specificity of primary antisera to the receptor of interest. Standard controls, such as omitting the primary antisera or preabsorbing the primary antisera with an excess immunising antigen, may still not confirm a high selectivity for the antibody. Current studies identifying the β -adrenoceptors in the bladder through immunohistochemistry will therefore require more stringent controls, such as the observation that the antigens lack binding sites in gene knockout animals, prior to a valid conclusion being presented.

Although β_2 -adrenoceptors appear to predominate the responses in rat and rabbit bladders (Morita et al., 1993), in guinea-pig detrusor the β_1 -adrenoceptor is the functionally active receptor subtype (Li et al., 1992, Yamamoto et al., 1998). The β_3 -adrenoceptor is thought to mediate relaxation in the dog bladder (Yamazaki et al., 1998). However, there have been conflicting results relating to the mouse bladder. One study published findings of an exclusively β_2 -mediated relaxation (Wuest et al., 2009), whereas another study found that relaxations occurred via the β_3 -adrenoceptor in this species (Deba et al., 2009). The conditions, concentrations and measurements used in these studies varied widely, such as the use of potassium chloride to pre-contract male detrusor strips, and using lower concentrations of antagonists (Wuest et al., 2009) vs electrical field stimulation of female detrusor strips, and higher concentrations of antagonists (Deba et al., 2009). However, the most notable difference was the age of the mice, with Deba *et al.* (2009) using mice that were 11 weeks old, where Wuest *et al.* (2009) used mice that were 9-18 months old. It is known that the expression of the β -adrenoceptors in humans is altered with age (Li et al., 2003), and a decreased response in the mature bladders may be related to the general decrease in β -adrenoceptor response which is known to occur during aging (Scarpace et al., 1991). Thus, although there were a range of differences in the methodology, the variations shown in the

two studies may infact be the first indication of a down-regulation of detrusor smooth muscle β -adrenoceptors in ageing mice.

β -adrenoceptors in the urothelium/lamina propria

The urothelial layer of the bladder expresses mRNA for all three β -adrenoceptor subtypes (Tyagi et al., 2009a, Otsuka et al., 2008, Ochodnický et al., 2012, Kullmann et al., 2011). Although β -adrenoceptor activation results in detrusor muscle relaxation (Deba et al., 2009, Otsuka et al., 2008), current research is yet to conclude whether the urothelium has an involvement in the bladder's overall responses to β -adrenoceptor stimulation. Tyagi *et al* (2009a) reported that the urothelium was not involved in the relaxation responses to isoprenaline in pig bladder. This was supported by another study on rat bladders, which did not find any difference to β_3 -adrenoceptor mediated detrusor relaxations with or without the urothelium/lamina propria (Kullmann et al., 2011). In contrast, a past study had shown a conflicting result, identifying effects of the urothelium in changing the concentration response curve to isoprenaline (a parallel rightward shift) in intact human bladder strips (with urothelium) as opposed to denuded detrusor (Otsuka et al., 2008). Although these studies varied in the use of different baselines (basal tension vs pre-contraction) prior to relaxation, and the obvious species differences, it still raises further questions as to the exact function of the urothelium in mediating these β -adrenoceptor relaxations.

Activation of the β -adrenoceptors on the urothelium of rats stimulates the release of nitric oxide, which could further enhance the relaxing effect of β -adrenoceptor agonists (Birder et al., 1998, Birder et al., 2002c). However, a study attempting to identify the role of the urothelium and lamina propria in mediating bladder responses to isoprenaline found that the

urothelium has a role in β -adrenoceptor stimulated inhibition of contraction, but not via the release of NO (Murakami et al., 2007). These authors attempted to identify the effect of β -adrenoceptor agonists on *in vitro* pig tissues that were pre-contracted with carbachol. However, this pre-contraction may not effectively represent normal conditions or functions of the bladder, where relaxation tends to occur during the filling stage, and contraction predominantly during the voiding phase. This data did present a novel mechanism of β -adrenoceptor mediated relaxation, as there are a range of similarities between human and pig β -adrenoceptor expression in the bladder (Goepel et al., 1997). In human detrusor a similar result was identified, which also found that the relaxing agent was not NO, giving rise to the hypothesis that β -adrenoceptor activation in the bladder may lead to the release of an unidentified inhibitory factor from the urothelium (Otsuka et al., 2008, Masunaga et al., 2010).

β -adrenoceptors as therapeutic targets in bladder disease

It is inconclusive as to whether the β -adrenoceptors are altered in pathological states of the bladder. Bladder outlet obstruction does not appear to influence the β -adrenoceptor response (Nomiya and Yamaguchi, 2003). Additionally, Igawa *et al.*, (2001) found that the β_3 -adrenoceptor was maintained as the predominant receptor involved in relaxation of both normal and neurogenic human bladders. However, the reliability of this study is questioned not only due to the fact that only 7 patients exhibiting overactive bladders were analysed, but that they also exhibited a varied aetiology of the neurogenic conditions. For example, 3 of the patients exhibited traumatic cervical cord injury, with 2 caused by hysterectomies and 1 of an unknown cause. This range of bladder pathologies may attest that each of these patients could have very different pathologies yet similar symptoms.

β_3 -adrenoceptor agonists are currently showing promise as clinical treatments for overactive bladder (Chapple et al., 2008). Solabegron, a β_3 -adrenoceptor agonist has been claimed to suppress the spontaneous activity of the human bladder, and inhibit evoked contractions (Biers et al., 2006a). Additionally another β_3 -agonist, mirabegron, has shown beneficial effects in animal studies where it decreases the frequency of spontaneous bladder contractions during the filling stage without affecting the amplitude during voiding (Takasu et al., 2007). Mirabegron has shown significantly positive effects in humans in a range of studies and as such, has been approved for clinical use in Japan (and recently submitted for approval in the United States and Europe) for the treatment of urinary frequency, urgency and incontinence associated with overactive bladder (Tyagi and Tyagi, 2010, Gras, 2012). Currently, although muscarinic receptor antagonists are the standard treatment for overactive bladder, they are limited by low response rates and frequent side effects (Andersson, 2004b). It is therefore important to identify alternative therapies. This current interest, focussed on the continued development and implementation of β -agonists for the treatment of overactive bladder, along with research into the effects of other β -agonists and antagonists on the urothelium/lamina propria, may provide a clearer understanding for the potential use of these drugs.

1.5.2.2 *α -adrenoceptors*

Subtypes of the α -adrenoceptor

Based on their anatomical position the adrenoceptors were originally divided into two subtypes, α and β (Ahlquist, 1948). This theory of two “sympathins” for the adrenoceptors was not only a breakthrough, it was one of the forefront discoveries which paved the way for a formal system of pharmacological taxonomy (Black, 1976). For nearly 30 years these two groups remained the predominate classification of adrenoceptors until presynaptic regulation of catecholamine release was studied and a classification for pre-junctional (α_2) and post-junctional (α_1) alpha-adrenoceptors was adopted (Langer, 1974). As pharmacological drugs with greater affinities were produced in the 1980’s, the α_1 -subtype was further divided into the α_{1A} , and α_{1B} . The onset of molecular cloning in the late 80’s and early 90’s allowed researchers to understand the genetic basis for these receptors, and an additional α_{1C} -subtype was presented (Schwinn et al., 1990). This finding, however, was later annulled, as the cloning, expression and tissue distribution of the rat homolog of the bovine α_{1C} -adrenergic receptor provided evidence that it was precisely the same phenotype as the α_{1A} subtype (Perez et al., 1994). A study which proposed an additional mechanism of action of the α_{1A} -subtype was later shown to be an independent receptor which was further labelled the α_{1D} (Lomasney et al., 1991, Perez et al., 1991). An additional subtype, labelled the α_{1L} -adrenoceptor, arises from the same gene as the α_{1A} , yet demonstrates functional differences, such as its low affinity for the antagonist prazosin, RS-17053 and WB4101, and its high affinity for tamsulosin (Gray and Ventura, 2006, Muramatsu et al., 2008, Daniels et al., 1999, Pennefather et al., 1999, Ford et al., 1996, Noble et al., 1997, Kava et al., 1998, Hiraoka et al., 1999). The α_1 -adrenoceptors stimulate contraction of smooth muscle in the lower urinary

tract through the phospholipase C dependent activation of IP3 and diacylglycerol. This leads to a calcium influx and subsequent activation of protein kinase C (Minneman, 1988).

Presence of α -adrenoceptors in the lower urinary tract

It is understood that although both α - and β -adrenoceptors are present in the ureters, noradrenaline acts predominantly through the α_1 -adrenoceptors, causing contractile responses across a range of animal models (Weiss et al., 1978, Morita and Suzuki, 1984, Labadia et al., 1987, Hernandez et al., 1992, McLeod et al., 1973). Adrenoceptors are also functionally active in the urethra where noradrenaline released from nearby nerves causes a contraction of the urethral muscle and is important for preventing urine leakage from the bladder. In addition, prejunctional α_2 -receptors may have the potential to influence the release of neurotransmitters from sympathetic nerves, yet there is no evidence that they play a role in regulating bladder function. In the prostate, the α_1 -adrenoceptors are known to mediate contractions, and are investigated due to their association in stimulating cell growth such as that seen in benign prostate hyperplasia (Kyprianou et al., 2000). The functional subtype mediating prostatic contractions consistently appears to be the α_{1L} -adrenoceptor, as seen in the mouse (Gray and Ventura, 2006), guinea pig (Pennefather et al., 1999), rat (Hiraoka et al., 1999) and human prostate (Muramatsu et al., 1994).

In the bladder, there have been some studies which have recognised the presence of α -adrenoceptors. mRNA analysis has identified the expression (yet not the translation) of α -adrenoceptors in whole monkey bladder tissue (Walden et al., 1997) and the α_1 -receptor subtype has been found in whole rat bladder using western blotting and immunohistochemistry (Ishihama et al., 2006). mRNA analysis has also identified their

presence in rat, mice and human bladders, and genetic analysis across species has found that the predominate subtype appears to be the α_{1A} -subtype (Malloy et al., 1998, Hampel et al., 2002, Lluel et al., 2003, Scofield et al., 1995). Studies have also reported expression of the α_1 -adrenoceptor protein in the bladders of rats, pigs, guinea-pigs, rabbits, cats, monkeys and humans (Hampel et al., 2002, Monneron et al., 2000, Tsujimoto et al., 1986, Latifpour et al., 1990, Walden et al., 1997, Malloy et al., 1998, Sigala et al., 2004). Nonetheless, it is still unclear as to their exact function within the bladder and the fact that they are expressed does not directly indicate that they have any important physiological roles. The α -adrenoceptor expression on spinal cord nerves innervating the bladder and the urethral sphincter give these receptors potential as therapeutic targets for treating urinary tract symptoms (Yoshimura et al., 2008b). The mRNA expression for the α_{1A} , α_{1D} and all subtypes of the α_2 -adrenoceptors have been located on human urothelium (Ochodnický et al., 2012). However, there is still a paucity of evidence for postjunctional α -adrenoceptors playing an important role in the function of normal bladder detrusor or urothelial/lamina propria activity.

Noradrenaline released from the hypogastric nerve may act upon the α -adrenoceptors present in the bladder outlet and urethra in order to maintain normal continence. A previous study found that α_1 -adrenoceptor agonists could enhance the release of acetylcholine and noradrenaline from bladder nerves (Somogyi et al., 1995). However, this group found that their presumed α_1 -adrenoceptor mediated response was not blocked by an α_1 -adrenoceptor antagonist, indicating that the released noradrenaline might be acting on β -adrenoceptors. The lack of distinctive physiological functions for the α -adrenoceptors in the bladder has caused them to be overlooked as possible therapeutic targets for alleviating the symptoms of bladder pathologies. A study using radioligand binding found that the α -adrenoceptor density on the bladder was not altered by age in rats (Kolta et al., 1984). Nonetheless, there are definite

age-related differences in the contractile responses to noradrenaline (Lluel et al., 2003) which may warrant further investigation into the role of the α -adrenoceptors in bladder disease.

α -adrenoceptors as a therapeutic target for lower urinary tract diseases

Throughout the past 20 years α_1 -adrenoceptor antagonists have proven an effective therapy for treating men with benign prostate hyperplasia (Lepor et al., 2012). A main target of this class of antagonists is the α_{1A} -mediated contraction of the smooth muscle in the prostate, and preventing this contractile activity helps to reduce the symptoms of prostate enlargement which subsequently constricts the urethra (Ventura et al., 2011). However, there is new clinical evidence that these α_1 -adrenoceptor antagonists may also act on other areas of the lower urinary tract, and alleviate other bladder malfunctions (Yoshida et al., 2012). Two studies in Japan have both identified decreases in pressure flow during urodynamic analysis on patients taking silodosin, an α_{1A} -selective adrenoceptor agonist (Yamanishi et al., 2010, Matsukawa et al., 2009). Matsukawa *et al.* (2009) also demonstrated the loss of detrusor overactivity in patients taking silodosin. These results indicate a potential additional role for the drugs, such as an influence on the detrusor smooth muscle or bladder dome. The effect of these drugs has been further demonstrated in a rat model, where a similar antagonist, tamsulosin, increased urine flow and reduced bladder overactivity (Okutsu et al., 2011). The potential for α_1 -adrenoceptor antagonists to reduce the symptoms of disorders such as overactive bladder is of considerable interest..

1.5.3 Purinergic receptors

Adenosine 5'-triphosphate (ATP) is a transmitter released throughout the body to evoke a broad spectrum of responses, including prejunctional modulation of neurotransmitter release and postjunctional receptor activation (Burnstock, 2011). In addition to being released from parasympathetic nerves, ATP is also known to arise from non-neuronal endogenous sources. Within the bladder, ATP acts upon the P2-purinoceptors. These have been subdivided into two groups, the P2X and the P2Y receptors based on pharmacological and molecular studies. P2X receptors are ionotropic ligand gated non-specific cation channels whereas the P2Y are G-protein coupled receptors (Burnstock, 2007).

Presence of the purinergic receptors in the bladder

There is evidence that a range of P2X and P2Y purinergic receptors are present in the lower urinary tract of both human and animal models. mRNA analysis has identified the P2X₁ as the predominant subtype (of the 7 characterised) within human detrusor muscle (O'Reilly et al., 2001b, O'Reilly et al., 2001a). Using animal models, immunohistochemical studies have suggested a similar finding in cat, mice and rat bladders (Birder et al., 2004, Lee et al., 2000, Vial and Evans, 2000). However, functional studies examining P2X₃-receptor gene knockout mice found that this receptor was essential for the sensory pain responses and afferent neuronal pathways controlling the micturition reflex (Cockayne et al., 2000). The sensory role may be mediated via the effect of ATP on the peripheral terminals of A δ and C bladder afferent nerve fibres, where it can convey mechanosensory information to the spinal cord (Ford et al., 2006).

A range of purinergic receptors are also present in the bladder urothelium (P2X₁, P2X₂, P2X₃, P2X₄, P2X₅, P2X₆, P2X₇, P2Y₁, P2Y₂, P2Y₄, P2Y₆) (O'Reilly et al., 2001a, O'Reilly et al., 2002, Sui et al., 2006). The presence of the P2X and P2Y purinergic receptor subtypes in the urothelium as well as on the lamina propria interstitial cells (Fry et al., 2012) and bladder nerves suggests that ATP may play a number of roles in the bladder (Birdier et al., 2004).

Purinergic responses in the bladder

Excitatory purinergic responses are found in the bladders of mammals such as rabbit (Dean & Dowie, 1978), guinea-pig (Burnstock et al., 1978), ferret, marmoset (Moss and Burnstock, 1985), cat (Theobald, 1983), rat (Burnstock et al., 1972) and mouse (Acevado & Contreras, 1985). ATP is understood to play a role in neuroeffector mechanisms and the human bladder responds to ATP with concentration-dependent contractions (Husted et al., 1983).

When applied intravesically into rat bladders, ATP causes an increase in the frequency of bladder contractions (Pandita and Andersson, 2002) identifying its potential importance in the modulation of contractile activity. ATP is released from the urothelium/lamina propria in response to stretch in strips of pig (Sadananda et al., 2009), rabbit (Ferguson et al., 1997), rat (Munoz et al., 2010, Sadananda et al., 2009), guinea pig (Young et al., 2012) and human tissues (Kumar et al., 2004). Additionally, urothelial/lamina propria ATP can be released in response to other stimuli, such as acidic solutions in pig bladder (Sadananda et al., 2012), and acid and activation of TRPV1 receptors in strips of rat urothelium/lamina propria (Sadananda et al., 2009). Urothelial cell cultures from samples of pig tissue have shown similar results, demonstrating a significant ATP release in response to acid and stretch (Cheng et al., 2011b). This has been further supported by studies on urothelial cell cultures from other animal

models, which also release ATP in response to stretch (Birder et al., 2003, Birder et al., 2002a).

Recent data from a guinea-pig model suggests that purinergic receptor mediated increases in bladder activity could occur through the P2Y₆ purinergic receptor subtype located on the interstitial cells (Sui et al., 2006). This conclusion resulted from observations of generated intracellular calcium transients. Due to the limitations of antibodies for these receptors, not all purinergic P2Y receptors were examined by the authors, leaving potential for other receptors to be involved. In addition, staining was also exhibited, albeit weakly, for the P2X₃, P2Y₂ and P2Y₄ receptors on these cells, but their function is unknown. Nonetheless, the data presented by Sui *et al.* (2006) has been supported by a more recent study with cultured interstitial cells from human lamina propria, where ATP was shown to significantly increase calcium activity, even at low concentrations (Cheng et al., 2011a).

Purinergic receptors as a therapeutic target in bladder disease

It has been predicted that the abnormal non-cholinergic bladder contractions associated with diseases such as overactive bladder demonstrate a degree of atropine resistance (Sjogren et al., 1982), and this may be mediated via ATP (Bayliss et al., 1999). The exact effect of purinergic receptor stimulation or antagonism in human patients is currently being investigated; although a lack of safe clinical purinergic agents limits current research. There is a possibility that patients with overactive bladder who do not respond to clinical antimuscarinics may have an increased sensitivity of the urothelium or detrusor to ATP; a heightened release of ATP from motor nerves or the urothelium; or reduced ATP hydrolysis within the neuromuscular junction. Of particular interest is the increase in ATP release in

rats with chemically-stimulated detrusor overactivity (Munoz et al., 2011). Bladder inflammation (Smith et al., 2005, Smith et al., 2008, Bodin and Burnstock, 1998), diabetes (Munoz et al., 2011) and spinal cord injury (Khera et al., 2004) have also been shown to enhance the release of ATP from bladders of rat models. Studies have shown that augmented ATP release from the urothelium, due to damage or pathology, activates P2X receptors on sensory nerves, causing painful sensations (Burnstock, 2001a, Burnstock, 2006). Additionally, studies on models of interstitial cystitis have demonstrated increases in stretch-invoked ATP release from urothelial cell cultures (Birder et al., 2003, Sun et al., 2001), as well as an alteration of the purinergic receptors in both the urothelium and detrusor smooth muscle (Birder et al., 2004, O'Reilly et al., 2002). This ATP release in response to noxious or mechanical stimuli may sensitise nociceptors in the urothelium, and be a contributing factor in diseases of the bladder. As such, the future development of effective purinergic antagonists may play an important role in the pharmacological management of lower urinary tract dysfunction.

1.5.4 Nitric oxide system of the bladder

Nitric Oxide (NO) is a nonadrenergic noncholinergic (NANC) inhibitory neurotransmitter in various sites around the body (Artim et al., 2009) where it signals a range of responses in different tissues (Schlossmann et al., 2000, Casteel et al., 2005, Feil and Kemp-Harper, 2006, Hofmann et al., 2006, Nishikimi et al., 2006, Åeckert et al., 2005). NO is a highly reactive chemical that holds an unpaired electron within its valence orbital and is non-polar enough to diffuse freely across membranes in the uncharged state. Due to its reactivity and toxicity, NO cannot be stored in the nerve terminals themselves but is synthesised from L-arginine by nitric oxide synthase.

Although the main neurotransmitter acting on detrusor smooth muscle appears to be acetylcholine (Sellers and Chess-Williams, 2012), the release of NO may also assist with normal bladder function. In the bladder, nitric oxide acts via the cyclic 3',5'-guanosine monophosphate (cGMP) pathway by activating soluble guanylate cyclase (Andersson and Persson, 1994, Caremél et al., 2010, Hernandez et al., 2008), which is similar to its effects in the nervous and cardiovascular systems (Moncada et al., 1991, Dinerman et al., 1993, Kerwin Jr et al., 1995). The production of nitric oxide is catalysed by a family of enzymes termed the nitric oxide synthases. Three isoforms of nitric oxide synthase (NOS) exist which are classified as inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) (Figure 1-13). nNOS was previously considered to be the main isoform responsible synthesising nitric oxide in the bladder tissue. However, it is now understood that endothelial nitric oxide synthase is also present in the bladder smooth muscle itself. In addition to eNOS, there is also evidence for the existence of mitochondrial NOS (mtNOS) in urothelial cells, which are modified analogues of the nNOS neuronal form (Kanai et al., 2004). The importance of each NOS isoform in bladder function is still under investigation (Zhang et al., 2011). Past studies have shown that mice lacking nNOS exhibited hypertrophy of their bladders (Burnett et al., 1997), while other similar studies have found no particular differences between those mice and wild-type controls (Sutherland et al., 1997). High concentrations of nNOS positive nerves have been identified in the bladders of human (Ehren et al., 1994), rat (Orman et al., 2005), pig (Persson et al., 1993) and guinea pig (Gillespie et al., 2005). These nerves may act directly on the detrusor, the urothelium/lamina propria, or the outflow region (trigone and urethra) of the lower urinary tract. This outflow region demonstrates a particularly strong relaxation response to NO (Torimoto et al., 2004, Masuda et al., 2003, Takeda et al., 1995, Martinez-Saenz et al., 2011). In the bladder neck, nitric

oxide is the main NANC mediator causing relaxation (Martinez-Saenz et al., 2011, Hernandez et al., 2008) and an open bladder neck is strongly correlated with the presence of stress urinary incontinence (English et al., 1999).

Various studies have identified a role for NO in the relaxation of bladder detrusor (Chung et al., 1996, Moon, 2002). Besides detrusor relaxation, NO may play an additional role in the regulation of blood flow within the bladder, as identified in canines (Pontari and Ruggieri, 1999) and rabbits (Whitbeck et al., 2007). Furthermore, in rat bladder smooth muscle cells the rate of protein and DNA synthesis was reduced after the addition of NO (Johansson et al., 2002). These authors utilised immunohistochemistry and western blot analysis, along with measuring the conversion of radiolabelled arginine, and suggested a role for nitric oxide as a growth inhibitory factor. This suggests that NO may have an additional role in regulating the growth of bladder smooth muscle cells.

Immunohistochemistry of the urothelium of the guinea pig bladder has detected the presence of nNOS (Gillespie et al., 2005), yet in the rat urothelium RT-PCR studies did not identify the expression of the nNOS gene (Birder et al., 2002c). A number of factors are known to cause the release of NO in the urothelium including β -adrenoceptor agonists, TRPV1 agonists, ATP and muscarinic receptor stimulation (Birder et al., 1998, Birder, 2011). In past studies this NO release has been detected using different methods, including measurements of cAMP accumulation, or by the use of a NO-specific sensor (Birder et al., 2002c). The effect of this urothelial-released NO on the bladder as a whole is currently under investigation. NO appears to have direct effects on the detrusor muscle to inhibit tonic contractions induced by carbachol (Moon, 2002) and this is mediated by the activation of potassium channels via a pathway involving cGMP (Deka and Brading, 2004).

Nitric oxide may also act on the interstitial cells in the lamina propria to increase cGMP levels within these cells (Gillespie et al., 2004). Alternatively, there has been limited data suggesting an excitatory role for nitric oxide/cGMP in bladder contractions (Gillespie and Drake, 2004, Moon, 2002, Fujiwara et al., 2000), further complicating the potential role that it may have in regulating bladder activity. In one study spontaneous phasic activity of the guinea-pig bladder was increased by the NO-donor nitroprusside and it was suggested that NO elevated cGMP in a subpopulation of interstitial cells within the lamina propria to induce microcontractions (Gillespie and Drake, 2004).

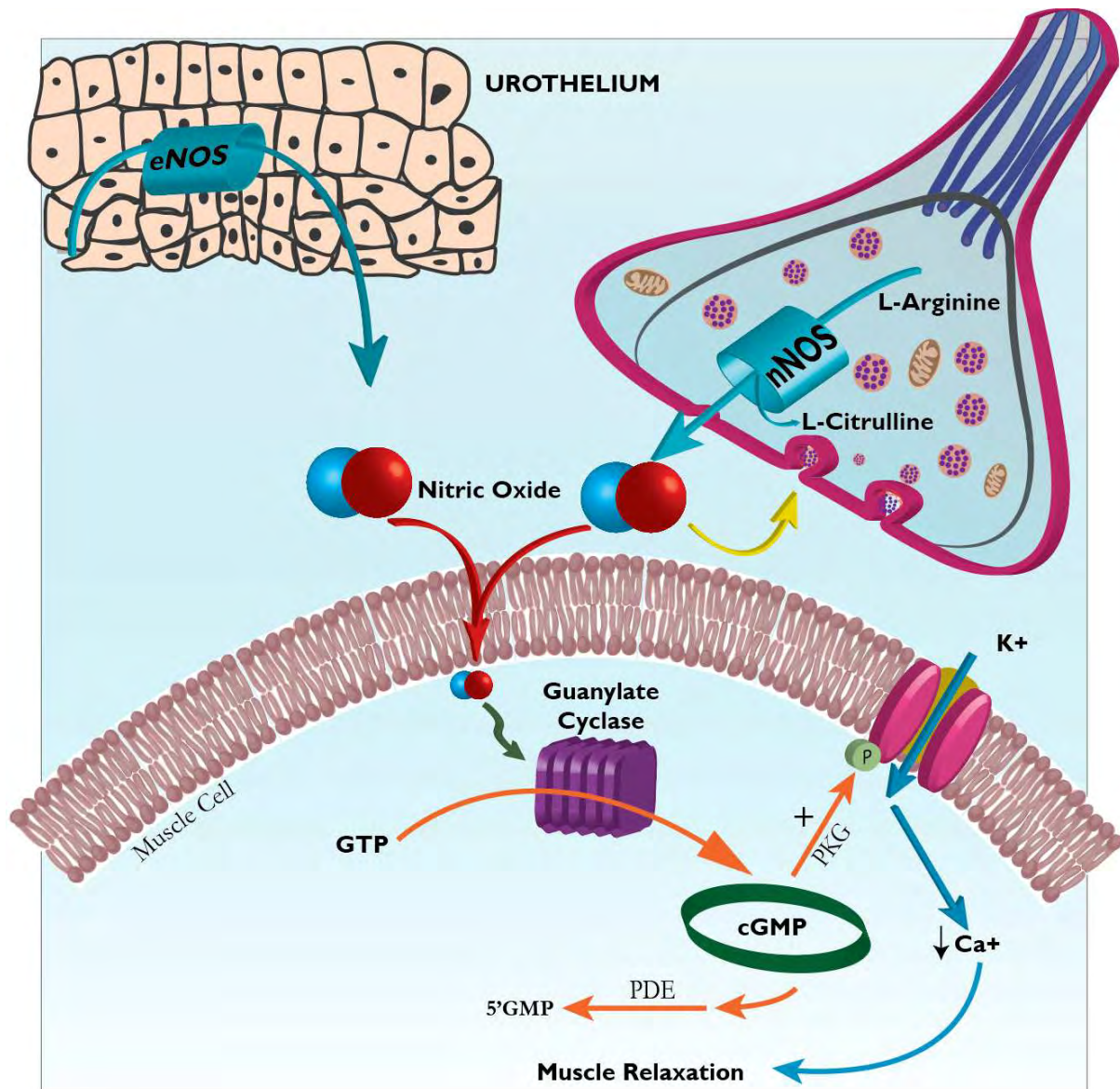


Figure 1-13: The synthesis of nitric oxide occurs within the urothelium and nerve cells innervating the bladder. In the muscle cell, NO activates the guanylate cyclase-mediated synthesis of cyclic guanosine monophosphate (cGMP) from guanosine-5'-triphosphate (GTP). This molecule then interacts with a number of mediators including protein kinase G (PKG), ion channels, and a range of potential phosphodiesterases (PDE), which are most likely PDE 1 or 4 (Andersson et al., 2007, Uckert et al., 2006, Masuda, 2008).

1.5.5 Cannabinoid receptors

A clear interest in cannabinoid receptor function in the bladder arose from studies where human patients found beneficial effects towards symptoms of bladder disorders following administration of cannabinoid agonists. These benefits included alleviation of urgency, frequency, nocturia and incontinence episodes, while being administered cannabis extracts (Freeman et al., 2006, Russo and Guy, 2006, Kavia et al., 2010, Perez, 2006, Brady et al., 2004). A survey of multiple sclerosis patients who commenced the smoking of cannabis, found that over 59% of patients reported improvements in their urinary urgency, incontinence episodes or hesitancy in initiating micturition (Consroe et al., 1997). In support of these studies, rats with partial urethral obstruction who were administered cannabimimetic, a selective cannabinoid-2 agonist, demonstrated an improved ability to empty the bladder (Gratzke et al., 2011). However, not all studies support this conclusion, such as the lack of beneficial effects observed on bladder activity after the administration of cannabis extracts to humans with bladder dysfunction as a result of multiple sclerosis (Wade et al., 2004). Additionally, limited effects have been observed with cannabinoid agonists on normal human bladder function, contraction and urodynamics (Gratzke et al., 2010, Freeman et al., 2006, Kavia et al., 2010). Nonetheless, cannabinoids may indicate an interesting avenue for future research into disorders of the urinary bladder, and their perceived successes in patients may designate them as a potential pharmacological target in future treatments.

Cannabinoid-receptors are found on nerves throughout the body. Both CB1 and CB2 receptor subtypes have been identified in the bladder detrusor and urothelium/lamina propria of humans via mRNA analysis (Tyagi et al., 2009b) and in the mouse bladder via western-blotting and mRNA analysis (Walczak et al., 2009). Both CB1 and CB2 are present in all

layers of the bladder as identified using immunohistochemistry in rat (Hayn et al., 2008), human (Tyagi et al., 2009b), monkey (Gratzke et al., 2009) and mouse (Walczak et al., 2009). However, caution must be taken when analysing these conclusions. In studies utilising immunohistochemistry, the specificity for antibodies used in the detection of the cannabinoid receptors have not been proven as selective for the individual subtypes (Ruggieri, 2011). These antibodies have come under the same scrutiny as those for other G-protein coupled receptors, and this lack of selectivity may explain why there is a discrepancy over which cannabinoid receptor is present in the neuronal cells of the bladder, with immunohistochemical reports identifying either a predominant CB1-receptor subtype (Hayn et al., 2008, Tyagi et al., 2009b, Walczak et al., 2009), or CB2-receptor (Gratzke et al., 2009).

Cannabinoids activate the CB1 and CB2 receptors, which couple to $G_{i/o}$ proteins, and result in an inhibition of adenylyl cyclase (Peters and Scott, 2009, Sade et al., 2006, Cabral, 2006, Mackie and Stella, 2006). Activation of CB1 receptors opens potassium channels and inhibits calcium channels. This activation presents a novel mechanism which may assist in the alleviation of lower urinary tract diseases (Ruggieri, 2011). However, the findings from functional studies investigating cannabinoid agonists are further complicated by the ability of the cannabinoids to have additional functions and activate other receptors. These include the TRPV1, which are present on the urothelial cells; and some effects that were originally attributed to CB2 receptor activation, and its related second messenger systems, may be due to one of these other systems (Gratzke et al., 2009).

The cannabinoids may have a regulatory function in maintaining bladder spontaneous contractile activity. Functionally active CB1-receptor subtypes (Tyagi et al., 2009b) might modulate the afferent activity of the bladder nerves (Walczak and Cervero, 2011, Walczak et

al., 2009). Administration of the cannabinoid receptor agonist WIN 55,212-2 reduced bladder motility in the rat, which was investigated through cystometric measurements (Dmitrieva and Berkley, 2002). In studies where neurotransmitter release was evoked by electrical field stimulation (EFS), cannabinoid antagonists have been shown to reduce the subsequent smooth muscle contraction in mouse, rat, pig, donkey, monkey and human bladders (Pertwee and Fernando, 1996, Martin et al., 2000). However, Martin *et al* (2000) found variations in this response between species and suggested there may be pharmacological differences between orthologues of the CB1 receptor, or even a mixed population of CB1 and CB2 receptor subtypes in different species. Although their roles are still unclear, cannabinoids warrant further research in their ability to alleviate the symptoms of bladder disease, and they are commonly identified as a potential therapeutic target for the management of lower urinary tract dysfunction (Apostolidis, 2012).

1.5.6 Transient receptor potential (TRP) receptors

Mammals have 28 genes encoding transient receptor potential ion channels (TRP receptors). The family includes canonical or classical (TRPC) vanilloid receptors (TRPV 1-6) and melastatin receptors (TRPM 1-8) (Hicks, 2006). These receptors have a diverse range of physiological functions which includes temperature transduction and signalling of pain (Eid, 2011). In current bladder research, the receptors of most interest are the TRPV receptors (Birder, 2005, Birder, 2007), which appear to respond to a range of stimuli, including pH, capsaicin, resiniferotoxin, lipids, and thermal stimulation (Kullmann et al., 2009, Everaerts et al., 2010). At the molecular level the genes for the TRPV1 receptors are expressed in the pig bladder urothelium/lamina propria as well as the detrusor in the dome, trigone and bladder neck (Sadananda et al., 2012). Immunohistochemical staining has also identified TRPV1

receptors expressed on the small diameter C-fibre afferent neurons which extend into the urothelium/lamina propria (Avelino et al., 2002, Yu et al., 2011a). A study using TRPV1 knockout mice have identified that these receptors play a clear role in the excitability of low threshold bladder afferent nerves (Daly et al., 2007). Other TRP receptors (TRPV1, TRPV2, TRPV4, TRPM8) are also present in the urothelium of a range of animal models (Birder et al., 2002b, Stein et al., 2004, Birder, 2005, Xu et al., 2009). However, not all studies have demonstrated clear expression of these receptors. In mouse bladder urothelium the PCR products for TRPV1 were minimal, and there was no evident staining for its protein expression (Yamada et al., 2009, Everaerts et al., 2010).

Upon activation, the TRPV channels may mediate bladder sensory responses (Mukerji et al., 2006a). Stimulation of the TRPV1 receptors on the urothelium/lamina propria can lead to release of transmitters such as ATP (Sadananda et al., 2009). Of further interest, is the possibility that the TRPV receptors are involved in bladder disease (Birder, 2007). For example, there is an increased expression of TRPV1 mRNA in human trigonal urothelium/lamina propria associated with sensory urgency (Liu et al., 2007). Additionally, the TRPV2 transcripts may play a role in controlling cell growth and the progression of bladder cancer (Yamada et al., 2010, Caprodossi et al., 2008). Furthermore capsaicin, a TRPV1-receptor agonist, can inhibit the growth of these tumor cells in the bladder by inducing apoptosis, identifying it as a possible chemotherapeutic agent for use in patients with bladder cancers (Yang et al., 2010, Lee et al., 2004). Of further interest is the finding that urothelial cell cultures from mice lacking the TRPV1 gene exhibit a reduction in stretch-induced ATP release (Birder et al., 2002b). TRPV6 was also considered as a mediator in bladder function, and has been thought to increase in prostate tumours, although no studies so far have found an upregulation of this receptor in bladder urothelial cancer (Santoni et al.,

2012). For now, the important research focus should be to understand the clear mechanisms behind the TRPV1-mediated effects in the bladder. There is a clear difficulty in interpreting results from this wide range of studies and although recent reviews have attempted to piece together the information (Yu and Hill, 2011), the roles for the functional TRP receptors will be an important area for future bladder research.

1.5.7 Neurokinin receptors

A range of neuropeptides are present in the bladder which may modulate or affect the activity of the urothelium and lamina propria. These include neurokinin A, substance P, calcitonin gene-related peptide, vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating peptide (Yoshiyama and de Groat, 2008, Arms and Vizzard, 2011, Warner et al., 2002, Smet et al., 1997). Importantly, neurokinin A has been singled out as a known mediator of urothelial/lamina propria contraction (Sadananda et al., 2008) and it is thought that neurokinins play an important role in regulating normal bladder activity (Keast and De Groat, 1992). Neurokinins are a class of peptide that bind to tachykinin receptors. Three of these receptors are expressed in the bladder, the neurokinin 1 receptor (NK1R), neurokinin 2 receptor (NK2R) and the neurokinin 3 receptor (NK3R) (Arms and Vizzard, 2011). These receptors are functionally active in the lower urinary tract and are preferentially stimulated by substance P (NK1R), neurokinin A (NK2R) and neurokinin B (NK3R) respectively (Andersson, 2002). Despite their selectivity, none of these agonists are specific for one receptor subtype and all neurokinins can act at each receptor, albeit at a slightly lower potency, giving them common mechanisms of action.

The neurokinin receptors contain 7 transmembrane spanning domains and each is coupled to the G-protein $G_{q/11}$, which activates the phosphoinositol pathway. The neurokinins that activate these receptors are released from the peripheral terminals of bladder afferent nerves ($A\delta$ -nerves). Both NK1R and NK2R are located in the detrusor (Birder, 2010). In the urothelium, low levels of the NK1-receptor can be seen at the mRNA level and by immunohistochemistry, yet the prevalence appears to be increased after capsaicin treatment (Heng et al., 2011). NK2 is also known to be present in the urothelium and its activation in both the detrusor and urothelium/lamina propria results in tissue contraction (Sadananda et al., 2008, Ishizuka et al., 1995, Templeman et al., 2003). The NK3R is expressed in only small amounts in the rat bladder (McCarson, 1999); although there is no functional evidence for its role in pig, human (Templeman et al., 2003), rat (Hall et al., 1992) or guinea pig (Longmore and Hill, 1992).

Interest has developed in the role of the NK1R / substance P system in the bladder, since it may be an underlying cause of interstitial cystitis via initiating neurogenic inflammation (Marchand et al., 1998, Saban et al., 2007, Ruggieri, 1998). The NK2R is also of particular interest, with its ability to elicit urothelium/lamina propria contractions (Sadananda et al., 2008). Abnormal expression or activation of the neurokinin receptors may be an underlying cause of bladder overactivity and other pathologies. In isolated strips of human detrusor, the NKA-induced contractile responses were depressed in samples of idiopathic overactive bladder, although not in neurogenic overactive bladder (Sellers et al., 2006). This further suggests a role in bladder disease, and further research into the neurokinin receptors of the bladder will provide a greater understanding of their significance.

1.5.8 Histamine receptors

The histamine receptor subtypes H₁, H₂, H₃ and H₄ have been shown immunohistochemically in human detrusor (Neuhaus et al., 2006). Functionally, histamine is known to cause contraction of the detrusor muscle *in vitro* (Poli et al., 1988, Rubinstein et al., 1987) where it may act directly on the muscle, or mediate the release of transmitters such as ATP or acetylcholine from nerves (Patra and Westfall, 1994). The histamine H₁-receptor subtype is recognised as the main subtype mediating contraction in the urinary bladder and when stimulated, is known to cause intracellular calcium release (Khanna et al., 1977, Neuhaus et al., 2006). However, Neuhaus *et al.*, (2006) also suggested a potential H₃-receptor subtype mediated effect, and further research into this receptor subtype might contribute to the overall knowledge of these receptors in the bladder. For now, the presence or functional role of the other histamine receptor subtypes in the bladder is not clearly understood.

There is a possibility that histamine receptors may have a role in bladder pathology. Release of histamine in the bladder can cause inflammatory responses as shown in guinea-pig tissues (Christensen et al., 1990), and abnormal concentrations of histamine release may occur in bladder diseases such as interstitial cystitis (French and Bhambore, 2011). This release of histamine potentially mediates the pain associated with the IC syndrome (Rudick et al., 2008). In addition, there is also the possibility that the urothelium can release an amount of histamine which is greater than the underlying detrusor release (Saban et al., 1994). These mechanisms of action for histamine in the lower urinary tract identify it as a potential therapeutic target for pathologies which induce sensory pain such as interstitial cystitis.

1.5.9 5-Hydroxytryptamine receptors

5-hydroxytryptamine (5-HT, also termed serotonin) is released from nerves innervating the lower urinary tract, and is a potential regulator of the micturition cycle (Ramage, 2006). This has led 5-HT modulators to be considered potential therapies for future treatments of lower urinary tract syndromes such as overactive bladder or stress incontinence (Tiwari and Naruganahalli, 2006, Vij et al., 2010). Their importance is highlighted by duloxetine, a 5-HT reuptake inhibitor used clinically, which has been demonstrated to have favourable results in alleviating stress incontinence in women (Basu and Duckett, 2009). Duloxetine was originally developed as an antidepressant, and it also affects the uptake of noradrenaline, yet it appears to increase bladder capacity and enhance urethral striated muscle activity in both animal models and human studies via the 5-HT system (Thor and Katofiasc, 1995, Duckett et al., 2008, Bump et al., 2008). In addition, recent trials have found positive results, greater than the placebo, for the use of this drug in overactive bladder and mixed urinary incontinence (Bent et al., 2008, Steers et al., 2007).

There are 14 structurally different 5-HT receptors, which have been classified into seven families (5-HT₁₋₇) based on their pharmacological properties (Ramage, 2006, Andersson and Pehrson, 2003). There is the potential for 5-HT to act both prejunctionally and postjunctionally within the bladder. Nerves in the mouse and rabbit bladder respond to 5-HT via prejunctional excitatory 5-HT₃ receptors (Chetty et al., 2007, Barras et al., 1996, Chen, 1990). Postjunctionally, the receptor mediating functional effects appears to be species dependent. Preparations of detrusor smooth muscle contract in response to 5-HT₂ receptor stimulation in human (Klarskov and Horby-Petersen, 1986), dog (Cohen, 1990), cat (Saxena et al., 1985) and rat (Kodama and Takimoto, 2000). Furthermore, the addition of 5-HT

causes a potentiation of neurogenic contractile responses in the urinary bladders of human (Hindmarsh et al., 1977), mouse (Holt et al., 1986), guinea pig (Messori et al., 1995) and rat (Rekik et al., 2011, Palea et al., 2004). The receptor mediating this potentiation response has been further identified as the 5HT₄-histamine receptor subtype in the pig (Sellers et al., 2000a) and human (Tonini et al., 1994, Chapple et al., 2004). However, in the cat it appears to be partly mediated via the 5HT₃-receptor subtype (Saxena et al., 1985). These mechanisms and functional effects demonstrated for the range of 5-HT receptors may present an interesting target for future therapeutic treatments of bladder pathologies.

1.5.10 Oestrogen receptors

The extent to which oestrogen affects normal bladder activity is unknown. Bladder diseases are thought to be more common in women and with age, which may relate to a correlation between their oestrogen levels and the mature onset of lower urinary tract disorders (Staskin, 2005, Miodrag et al., 1988). After menopause, for example, the lack of oestrogen appears to increase urethral tone (Rizk, 2005, Game et al., 2008). The mechanism for this is not well understood, yet in a past study increased levels of oestrogen, through frequent injections in rabbits, resulted in increases to the immunohistochemical staining for the adrenergic innervation of the detrusor (Levin et al., 1981). In addition, this study identified increases in the responses to both adrenoceptor and muscarinic receptor agonists. This correlates with a more recent study suggesting that oestrogen may be responsible for reducing the release of acetylcholine from efferent nerves (Yoshida et al., 2007).

Oestrogen is a key modulator of pain and dysfunction and as such, it is generally thought that reduced levels of oestrogen would impact a range of mechanisms within the bladder wall. There is evidence that suggests both beneficial (Miodrag et al., 1988, Cody et al., 2009) and detrimental effects of oestrogen in lower urinary tract function (Jackson et al., 2002, Falconer et al., 1998, Keane et al., 1997, Palmer and Newman, 2007, DuBeau, 2005, Palmieri et al., 2007). Patients taking oestrogen in clinical trials of hormone-replacement therapy reported increases in both stress and urgency urinary incontinence (Hendrix et al., 2005, Townsend et al., 2009, Grady et al., 2001). Alternatively, when applied locally it appears that there is a reduction in the symptoms related to urinary dysfunction (Cody et al., 2009). In these studies, patient reports from those on hormone replacement therapy tend to contradict findings from the laboratory. However, it does appear that there are a range of alterations occurring during systematic oestrogen treatment. The potential benefits observed from local administration suggest a direct receptor-mediated effect on the lower urinary tract.

Within the bladder, the concentration of oestrogen receptors appears to be higher in the urethra when compared to the dome or trigone (Iosif et al., 1981, Robinson and Cardozo, 2011). Nonetheless, immunohistochemistry has demonstrated the presence of these receptors on the urothelium and therefore a potential mechanism involved in tissue function (Miyamoto et al., 2012). Oestrogen can regulate various visceral activities and influence a range of non-genomic mechanisms in the bladder, such as activation of TRPV1 receptors (Kiasalari et al., 2010). β -estradiol, acting on the E_2 -receptor appears to modulate kinase activity in rat urothelial tissue (Birder et al., 2010c) and detrusor tissue (Valeri et al., 2009). Oestrogen might even cause short term changes in bladder activity or increase the incidence of OAB in the female. Tamoxifen, an oestrogen receptor antagonist, or modulators of protein kinase C (which is necessary in mediating the effects of oestrogen) have been suggested as

preventative agents for increases in the oestrogen levels that may contribute to bladder diseases such as OAB (Robinson and Cardozo, 2011). Nonetheless, by itself oestrogen therapy does not appear to have a benefit to the management of bladder dysfunction. However, studies which have used oestrogen in conjunction with an α -adrenoceptor agonist have found that it may improve the leakage of urine in patients with stress incontinence (Robinson and Cardozo, 2003, Hilton et al., 1990).

Clear beneficial evidence for the therapeutic use of oestrogen is still lacking and there is no experimental or clinical evidence to suggest exactly what mechanisms could be targeted for oestrogen treatment (Rechberger and Skorupski, 2007). Although oestrogen levels may be a contributing factor towards stress incontinence in post-menopausal women, there is controversy regarding the effectiveness of oestrogen supplementation as an effective treatment (Koski and Chermansky, 2011, Robinson and Cardozo, 2011). Additionally, in males a range of data indicates that exposure to oestrogen therapy may cause hyper-proliferative or inflammatory responses in the prostate and obstructive voiding later in life (Li and Rahman, 2010). This further identifies the caution required when examining or recommending oestrogen supplementation as a treatment for lower urinary tract dysfunction. Nonetheless, it may be important for further research to identify the effects of oestrogen on the functions of other receptors in the lower urinary tract, in an attempt to fully understand the mechanisms underlying its effects.

1.6 DISORDERS OF THE BLADDER

1.6.1 *Overactive bladder*

The overactive bladder syndrome is characterised by patients experiencing urinary urgency, with or without urge incontinence, and is usually coupled with frequency and nocturia (Abrams et al., 2002). Studies in Western Europe and the USA have found that up to 17% of adults are affected by an overactive bladder (Stewart et al., 2003, Milsom et al., 2001). Patients experience a degree of urinary urgency and although it does not always result in urinary incontinence, 30% of sufferers experience involuntary leakage (Milsom et al., 2001, Milsom et al., 2000, Stewart et al., 2001, Stewart et al., 2003). Frequency of greater than 8 voids per day is usually required and nocturia is commonly present (Rovner and Wein, 2003). This inability to hold urine long enough to reach a restroom, including having intermittent strong desires to urinate causes a reduction in the quality of life in sufferers (Kelleher, 2002).

A malfunctioning urinary bladder as a result of neurological dysfunction, injury or degeneration of the neural pathways controlling micturition is termed a neurogenic bladder (Ashok and Wang, 2010). The symptoms of neurogenic bladder can involve detrusor underactivity or overactivity depending on the site of neurological insult. Supraspinal lesions of the central nervous system above the pontine micturition centre, in the brainstem, leads to a loss of voluntary control of urination. Studies on rats have demonstrated that cerebral damage can reduce central inhibition of the detrusor muscle, leading to potential bladder overactivity and a significantly smaller bladder capacity (Yokoyama et al., 1998b, Yokoyama et al., 2000). From these studies on rats it appears that the dopaminergic and glutamatergic pathways contribute to bladder overactivity following brain lesions (Yokoyama et al., 1999, Yokoyama et al., 1998a), although research is yet to confirm this in humans. The reduction

in bladder capacity and increased bladder overactivity is not limited to cerebral lesions as haemorrhages, tumours, dementia, and other cerebral diseases are also known to cause similar symptoms. For example, the central nervous system in patients with Parkinson's disease exhibits a degeneration of the basal ganglia, which often results in patients displaying signs of bladder overactivity during the filling stage (Uchiyama et al., 2011).

Neuronal trauma below the brain, at the level of the spinal cord, is also associated with a loss of control in the urination reflex. This is due to inhibited communication between the bladder and the pontine micturition centre. Facilitation or inhibition of the detrusor contractions can occur after lesions in several areas of the central nervous system. This has been supported historically through a range of animal studies and clinical evidence in humans (Andrew et al., 1966, Andrew and Nathan, 1964, Barrington, 1927, Bors, 1952, Sherrington, 1892, Satoh et al., 1978, Nadelhaft and Booth, 1984). In addition, changes in the activity of bladder afferent nerves have been demonstrated following spinal cord injury in animal models (de Groat et al., 1990, de Groat et al., 1997). However, it is still unclear as to exactly what changes occur after spinal cord injury that would specifically induce overactivity in humans. Other neurogenic causes of overactive bladder may be associated with damage to the neurons innervating the bladder hence resulting in the loss of nervous control.

Some patients have an overactive bladder as a result of *detrusor overactivity*. This is a clinical urodynamic observation characterised by involuntary detrusor contractions throughout the filling phase, which may be spontaneous or provoked (Abrams et al., 2002). There is a correlation between patients that have detrusor overactivity and overactive bladder symptoms (Al-Ghazo et al., 2011). Urine can leak due to these abnormal spontaneous bladder contractions while the bladder is filling. Animal models of neurogenic OAB,

obstructive OAB and OAB associated with diabetes show some of the presentations commonly seen in patients with detrusor overactivity. These models display a super-sensitivity of the muscle to agonist stimulation suggesting that detrusor myocytes become more responsive to electrical signals (Vahabi et al., 2011, Vahabi et al., 2010, Stevens et al., 2006, Brading, 1997).

Although there are clear neurogenic origins for some sufferers of detrusor overactivity, idiopathic causes represent the largest population of patients. The mechanisms underlying this form of overactivity are unclear but appear to involve both changes in neurogenic (neurological innervations to the bladder) and myogenic (smooth muscle) activity. It is understood that dysfunction in either of these systems may give rise to overactive bladder.

1.6.2 Interstitial cystitis

Interstitial Cystitis (IC) is commonly termed painful bladder syndrome. It is broadly described by an unpleasant sensation such as pain, pressure or discomfort perceived to be related to the urinary bladder and cannot be attributed to other causes (Hanno et al., 2011). Interstitial cystitis has not been linked to any known infection of the bladder. It was traditionally diagnosed in patients suffering from lower urinary tract pain and dysfunction that cannot be classified under any of the normal bladder diseases (detrusor overactivity, bacterial cystitis, etc). Along with the localised discomfort associated with having a painful bladder, many sufferers of IC report additional pain in the lower abdomen, lower back and urethra. Diagnosis of IC in patients tends to occur after bladder pain or irritation has persisted for longer than 6 months (Hanno et al., 2011). Following this initial diagnosis there is currently no universally successful treatment reported for this disease.

Interstitial cystitis is a multifactorial disease whose symptoms have similarities with those of the overactive bladder syndrome such as urinary urgency, frequency and dysuria. The onset of chronic pelvic pain, however, is the symptom that separates it from other disorders. Of these symptoms, it is the chronic pelvic pain that is the most difficult to control. The current understanding of the aetiology of IC is incomplete and a variety of mechanisms may be involved at different stages of the disease. With common misdiagnoses it is not known how many people suffer from IC, with reports ranging from less than 1% to as high as 11% of females (Berry et al., 2010). However, the diagnoses for IC are broad and the prevalence of IC detected by urologists may simply depend on the definition used to identify it. The lack of clinical understanding for its management is evident from a study investigating the treatments prescribed to women with IC found 183 different types of therapies recommended by doctors (Rovner et al., 2000).

Studies have suggested a range of bacterial infections (both gram-negative and gram-positive) which can cause bladder inflammation (Echols et al., 1999). However, cases of interstitial cystitis do not have any underlying infection, signifying that the inflammation is either due to infections which are difficult to test for, such as unknown pathogens or non-infectious causes. Known causes of inflammation in other areas of the body include exposure to radiation, medication side-effects, chemicals, autoimmune responses and cancer. However, the extent of how these may contribute to the chronic effects seen in IC patients is unknown. It has been suggested that IC may relate to the activation of mast cells (Sant and Theoharides, 1994, Theoharides et al., 2001), increases in substance-P positive sensory nerve fibres (Pang et al., 1995), abnormal release of histamine (el-Mansoury et al., 1994) or dysfunction of the sympathetic innervation (Peeker et al., 2000). Alternatively, a deterioration of the bladder's protective GAG layer may lead to symptoms of IC (Chiang et

al., 2000, Metts, 2001, Ruggieri et al., 1994, French and Bhambore, 2011). Treatments have attempted to replace this barrier with oral or intravesical pentosan polysulfate sodium or intravesical heparin or hyaluronic acid, which can assist with the repair of the GAG layer. Although there have been positive responses to this treatment in patients (Sand et al., 2008, Anderson and Perry, 2006), the broad spectrum of possible causes for IC means that patients who do not exhibit a reduction in symptoms from these treatments may have alternative aetiologies and this treatment may not be effective in a number of IC cases (Nordling, 2004, Sant et al., 2003). Recent findings have also linked IC with recreational abuse of the drug *ketamine* (Nomiya et al., 2011, Chen et al., 2009, Chu et al., 2008) although no clear hypothesis was provided to suggest how this chemical causes damage to the bladder and induces chronic inflammation.

Alternatively, the fact that pain is prevalent across areas such as the lower back and abdomen means that the symptoms shown from IC may not even arise from the bladder. The pressure and burning sensations may indicate abnormal functioning of the myelinated C-fibres which connect to the colon as well as the bladder. The wide range of symptoms makes the profile of IC difficult to target and correctly diagnose (Whitmore and Theoharides, 2011). To advance the ability to treat IC a range of measures needs to be taken. Rather than focussing solely on pharmaceutical or surgical treatments, research is required on behavioural, diet and lifestyle alterations which might alleviate the cause. It has been shown that behavioural modifications, such as bladder training and monitoring of voiding intervals, demonstrate a 50% reduction in IC symptoms (Parsons and Koprowski, 1991). Further scientific studies on the benefits of diets and other common treatments in alleviating IC are required. This research data would assist physicians to correctly diagnose and give evidence-based

prescriptions to patients rather than the current excessive range of widely ineffective treatments.

1.6.3 Stress incontinence

Stress incontinence is the most common form of incontinence in women. It involves involuntary leakage of urine during activities that increase abdominal pressure, such as coughing, sneezing or jogging. The symptoms of stress incontinence can be caused by a lowered bladder outlet resistance where urethral tone is reduced such as in old age, or by repositioning of the bladder due to a lack of pelvic floor support, as often found after pregnancy and childbirth. Surgical treatments for stress incontinence include Burch colposuspension, where an incision is made in the abdomen and permanent stitches (Burch sutures) are placed around the bladder, anchoring it to the pubic bone. The attachment to this bone at the lower part of the abdomen supports and assists the bladder to help prevent urinary leakage (Lapitan et al., 2009). Newer procedures such as laparoscopic colposuspension are similar although a number of smaller incisions are used rather than one large incision in the abdomen. Alternatively, a relatively new treatment involves insertion of a sub-urethral sling around the bladder neck which supports it during times of stress (Rehman et al., 2011). Nonetheless, these invasive surgery options are not practical for all patients and not always successful (Ogah et al., 2011, Sander and Lose, 2007). Non-surgical procedures include initial treatments such as remedial exercises that increase the pelvic floor muscles. This tends to be highly effective in reducing the prevalence of incontinence episodes (Dumoulin et al., 2011, Bo, 2004). In addition, lifestyle changes such as a reduction in weight; or lowering the intake of caffeine, alcohol or nicotine might also be effective in that they tend to alleviate the symptoms of stress incontinence in a number of patients (Jura et al., 2011, Townsend et al., 2008).

1.6.4 *Bladder infections*

Genitourinary infections are one of the leading causes of disease in humans, with urinary tract infections the most common complaint within this group (Schappert and Burt, 2006). These infections are usually associated with the bacterium *E-coli* often resulting in bladder inflammation (Nicolle et al., 2005, Hooton and Stamm, 1997, Kline et al., 2012). In response to bladder inflammation, patients can experience pain, urgency and an increased frequency of urination.

Attempts have been made to design effective animal models which can be used to simulate bladder inflammation (Bjorling et al., 2011, Hopkins et al., 1998, Bjorling et al., 2008). However, the relevance of these models is uncertain, due to the fact that responses to infection in human bladders are variable depending on the strain and severity of the invading bacteria and the individual's immune response. Animal models often use chemicals to irritate the bladder which result in chronic inflammation (Dinis et al., 2004, Krhut and Zvara, 2011, Cayan et al., 2003). However, it is uncertain how relevant this “chemical cystitis” is to natural symptoms of bladder inflammation in humans. Nonetheless, these models do present novel mechanisms underlying the disease which may shed more light on the onset of inflammation in human urogenital tracts.

1.6.5 *Overflow incontinence and bladder outlet obstruction*

Obstruction of the bladder outlet or a deficiency of the detrusor muscle to completely empty the bladder can cause symptoms of bladder outlet obstruction (BOO) (Drake et al., 2003d). This disorder can lead to the incomplete emptying of the bladder or over-distension resulting

in involuntary leakage due to the subsequent overflow (overflow incontinence). This problem is common in male sufferers of prostate hyperplasia as the detrusor muscle is not strong enough to completely void the bladder whilst the prostate forces pressure on the urethra. Catheterisation remains the predominate method for alleviating these symptoms. Other problems with bladder obstruction include the recurrence of urinary infections, weak urinary flow rates, terminal dribbling, difficulty in beginning the flow of urine (hesitancy), and an increase in the prevalence of overactive bladder.

1.6.6 Bladder dysfunction in Australia

Up to 15% of Australians and over 30% of elderly Australians are estimated to have some form of urinary incontinence (Pearson et al., 2002). It is assumed that only 31% of these sufferers report having sought help from a health professional (Continence foundation of Australia, 2010). The most common pharmacological treatments are clinical antimuscarinic drugs such as oxybutynin, tolterodine, darifenacin and solifenacin (Athanasopoulos and Giannitsas, 2011). These drugs have consistently demonstrated small, yet statistically significant differences in their efficacy and tolerability (Robinson and Cardozo, 2010). However, the antimuscarinics are known to bind to muscarinic receptors in other organs such as the salivary glands where they cause dry-mouth, resulting in a constant discomfort to patients. Additional actions on other areas in the body can produce further side effects including constipation, drowsiness, blurred vision, increased heart rate, dizziness and cognitive impairment (Saks and Arya, 2009).

The most common forms of urinary incontinence are associated with a combination of urgency and stress and the three categories are not necessarily encountered independent of each other (Figure 1-14). Australia already has 66% of nursing home residents requiring

some sort of bladder management (Pearson et al., 2002). The cost to the health care system will continue to increase with the ageing population, who tend to have a higher prevalence of incontinence and bladder diseases (Kelleher, 2002). Effective management of incontinence will assist in:

- Reducing the cost of hospital treatments. The estimated cost of incontinence in Australia is \$1.5 billion, and that cost is expected to rise over 200% by 2030.
- Freeing up nurses and hospital staff from cleaning up incontinence-related spills. Up to 60% of primary aid workers spend 40 hours a week or more managing patients with incontinence, and 44% of primary carers report a change in their physical or emotional wellbeing since taking on the caring role, compared to 27% of carers who did not usually assist in managing incontinence.
- Greatly increasing the quality of life for the large numbers of patients suffering from involuntary leakage of urine, with over 117,700 healthy life years lost per annum due to incontinence.

(Statistics supplied by the *Continence foundation of Australia, 2010*, the *Commonwealth Department of Health and Ageing, 2011* and the *Australian Institute of Health and Welfare, 2012*).

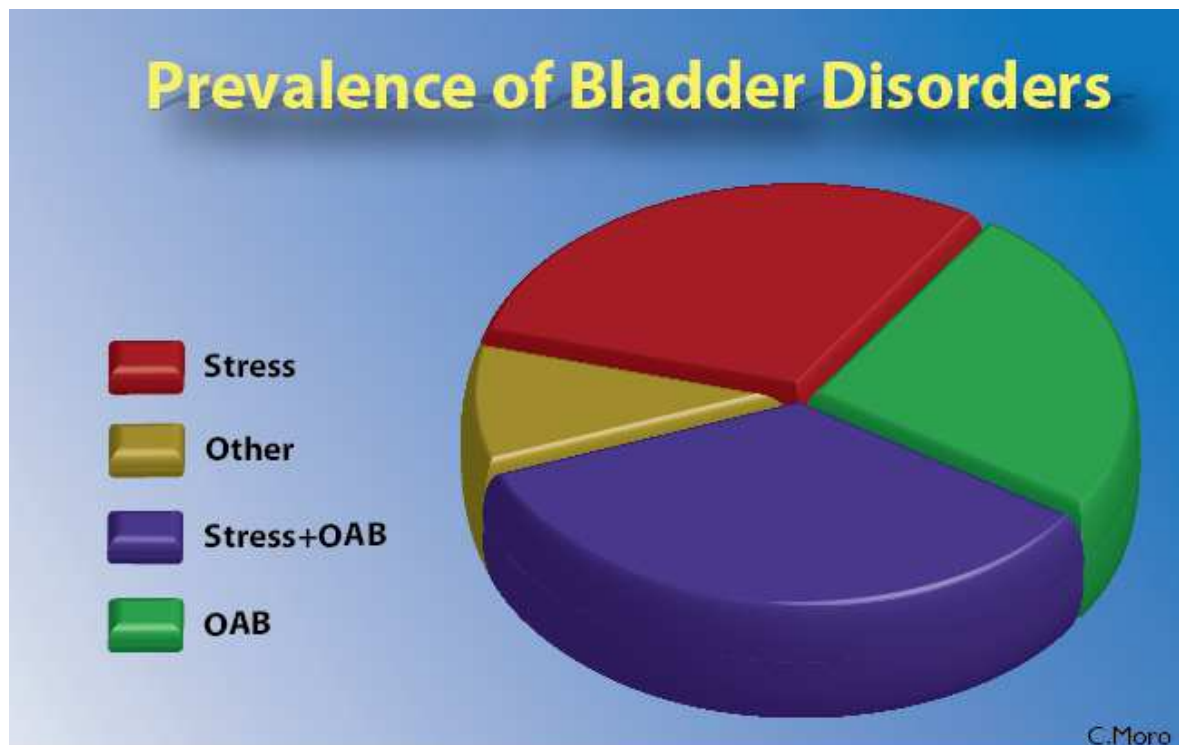


Figure 1-14: The prevalence of bladder disorders in Australia. The chart incorporates stress incontinence, other forms of incontinence (including overflow), Mixed (OAB + Stress incontinence), and isolated OAB cases (Australia, 2009, Pearson et al., 2002).

1.7 GENERAL HYPOTHESIS AND AIMS

The urothelium/lamina propria generates spontaneous contractions which are independent of any neuronal or detrusor activity and it is hypothesised that the receptors and transmitters, normally associated with the autonomic nervous system, regulates this activity. The objectives of this thesis were to investigate urothelial/lamina propria spontaneous activity and:

1. Measure the frequency, amplitude and baseline tension of spontaneous contractions.
2. Identify agonists and antagonists that modulate the spontaneous contractile activity.
3. Determine the effect of the nitric oxide system, and the muscarinic and adrenergic receptor subtypes which influence contractile rate and tension in response to stretch or receptor stimulation.
4. Determine the effect of nerve stimulation on contractile activity.
5. Identify the role of the isolated urothelium in mediating spontaneous contractile activity of the urothelium/lamina propria.



Chapter 2

MATERIALS AND METHODS

2.1.1 *Collection of bladders*

Pig bladders were used in this study as a suitable model to investigate human bladder function. It has been previously shown that the porcine bladder is structurally similar to the human bladder and shares similar physiology and pharmacology (Sibley, 1984, Crowe and Burnstock, 1989, Templeman et al., 2003, Buckner et al., 2000, Kumar et al., 2004). In addition, diseases prevalent in human bladders, such as detrusor overactivity have been exhibited in the pig (Jorgensen et al., 1983, Sibley, 1985, Speakman et al., 1987, Chapple and Smith, 1994). Highchester abattoir (Gleneagle, QLD) supplied fresh tissues several times each week.

Bladders were obtained from Large White-Landrace-Duroc crossbred pigs (*Sus scrofa domestica*), slaughtered for the provision of bacon. These bacon-pigs were chosen as they are consistent in their size, weight and age. Pigs of both sexes were used since initial preliminary experiments demonstrated no differences in resting frequency, baseline tension or amplitude of spontaneous contractile activity. Sows, suckling pigs and other ages, sizes and breeds were not used. The pigs were 6 months old and weighed ~100 Kg *live weight*, and ~80 Kg *dead weight*, after the removal of offal.

2.1.2 *Tissue preparation*

Following slaughter bladders were immediately immersed in cold Krebs-bicarbonate solution (composition in mM: NaCl 188.4, NaHCO₃ 24.9, CaCl₂ 1.9, MgSO₄ 1.2, KCl 4.7, KH₂PO₄ 1.2 and D-glucose 11.7) and transported to the Bond University laboratories on ice. The urethras and ureters were removed and the bladders opened longitudinally (Figure 2-1). Full

thickness strips of anterior wall from the dome region were removed. From these tissues, strips of urothelium including the lamina propria were prepared (2cm x 5mm). All serosa and surrounding tissue was removed from the strips. For smooth muscle experiments detrusor strips were examined following removal of the urothelium and lamina propria.

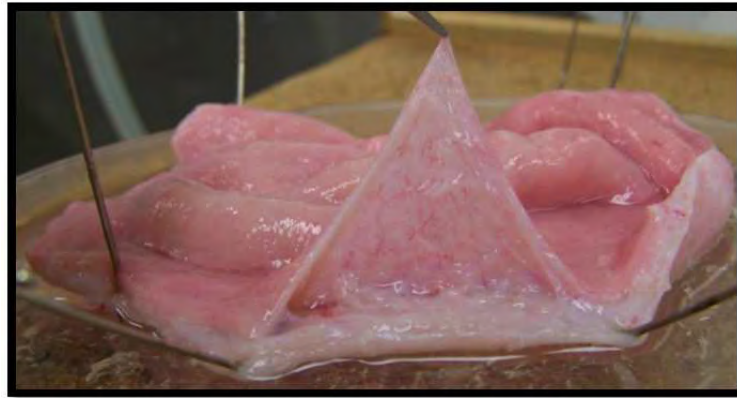


Figure 2-1: Whole pig bladder. The urothelial and lamina propria layers have been dissected away from the detrusor for inspection. Note the clear colour and texture difference between the underlying white detrusor muscle and the pink lamina propria layer. Through observation of this internal lamina propria layer, it is clear that the urothelium and lamina propria contains a rich network of capillaries and blood supply.

2.1.3 *Functional organ bath experiments*

Tissues were immersed in 10 mL organ baths in Krebs-bicarbonate solution (Labglass, Brisbane, Figure 2-3). Throughout the experiments tissues were maintained at 37°C and gassed (95% O₂, 5% CO₂). In each organ bath, tissues were attached to isometric force transducers (ADInstruments MCT050/D, Figure 2-2). A small syringe was used to anchor the tissue to the base of the bath, and cotton twine was used to connect the tissue to the transducer longitudinally. Developed tension was amplified using an octal-bridge amplifier (ADInstruments) and transmitted to the computer via a *Powerlab* analogue-to-digital recorder (ADInstruments, Castle Hill, Australia). Developed tension was converted into a graphical trace using Labchart v7 software.

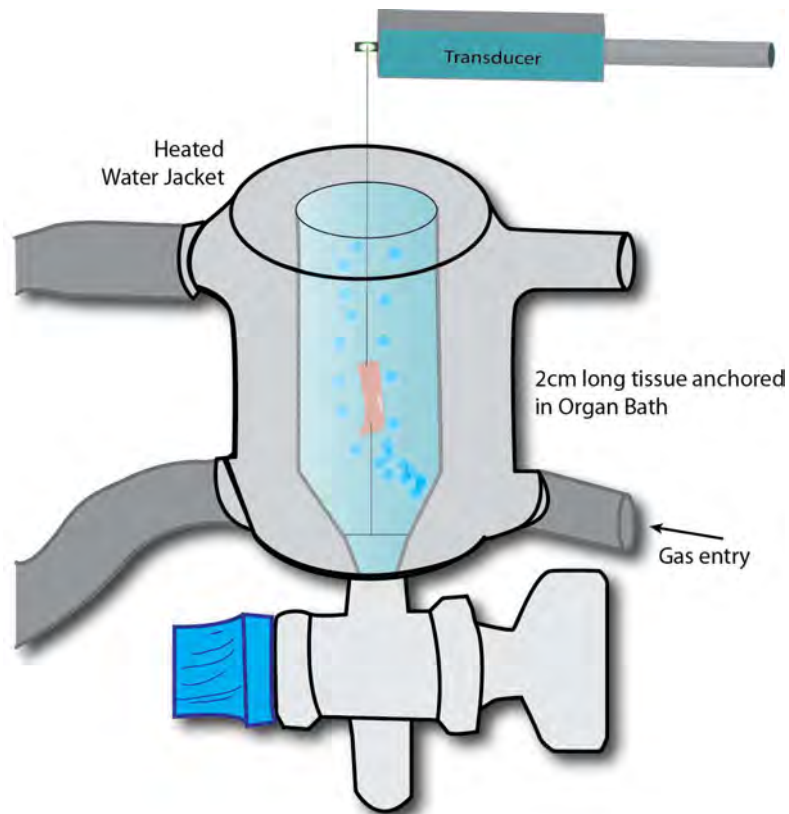


Figure 2-2: Custom-made organ baths (C. Moro, Labglass, Brisbane). The tissue was connected to a MCT050/D isometric force transducer, and contractile activity recorded through an octal-bridge amplifier connected to a Powerlab 8/30 system.

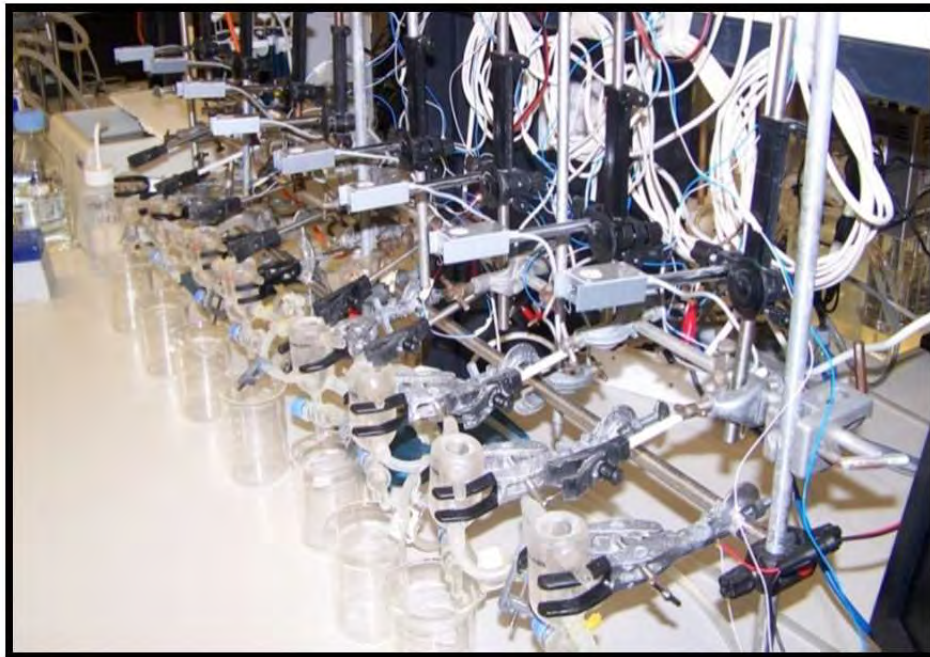


Figure 2-3: The experimental organ bath setup. The organ baths were designed to maintain internal temperature of 37°C with a constant and steady stream of carbogen gas.

2.1.4 Measurements of spontaneous activity:

Contractions were measured within a 2 - 5 minute period. The frequency over the time frame was converted to cycles per minute. The amplitude of spontaneous activity was measured as absolute values (g, Figure 2-4). Data was tabulated in Microsoft Excel 2010 and expressed as mean \pm SEM.

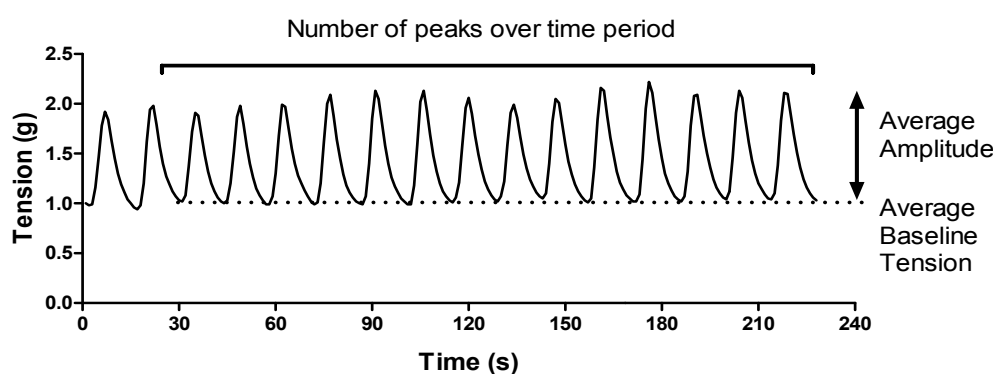


Figure 2-4: Calculation of the amplitude and the frequency of spontaneous activity.

Responses to agonists:

Measurements of frequency and amplitude of phasic contractions were taken at the peak response (Figure 2-5) after the addition of each drug or following stretching. The frequency of contractions was expressed as the number of phasic waves per minute (cycles min^{-1}) and the amplitude as grams tension. The baseline tension was taken as the lowest point of the spontaneous phasic contractions. After maintaining tissues under a baseline tension of 2g the tissues were allowed to equilibrate for 1 hour, with washes every 15 minutes with fresh warmed Krebs solution prior to the addition of any drugs. All antagonists were left to

incubate for at least 30 minutes with the tissues. In stretch experiments tissues were stretched in pairs (with or without the selective antagonist) by 1.5cm (75%) over 90 seconds and frequency was recorded before and after stretching.

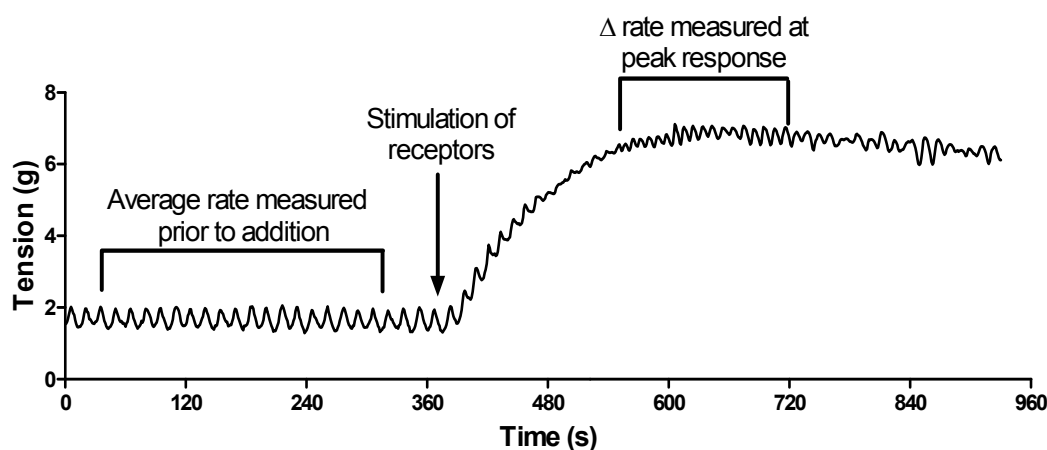


Figure 2-5: Demonstration of methods used to determine responses to agonists. Initial calculations were made after 40 - 60 minutes of the tissue equilibrating and a steady rate of spontaneous activity, baseline tension, and amplitude was evident. The average rate (in cycles second⁻¹) was measured within 3 - 4 minutes prior to the stimulation, then measured again for 2 - 3 minutes at the peak response to the agonist.

2.1.5 *Urothelial contractile responses: concentration-response curves*

Concentration-response curves to selected agonists were obtained by the addition of the drug to the organ bath in increasing increments. Most concentration-response experiments performed were within a range that did not exceed 0.05 - 300 μ M. Control experiments were performed with the addition of the vehicle alone and these experiments were used, if necessary, to correct for any time-dependent changes in tissue sensitivity or responsiveness during the course of the experiment. Each increment of the drug was added at peak tension response (Figure 2-5), which was usually within 4 minutes of the previous dose.

After the initial curve to maximum response, the tissue was washed out several times and left to return to baseline tension for 30 minutes. For studies involving agonists, the drug was added and the concentration-response curve completed from incremental drug additions. For experiments involving antagonists, drugs were added 30 minutes prior to the commencement of the second concentration-response curve in the presence of the antagonist. A maximum of 2 curves were completed for each tissue. In experiments where a second curve was not possible due to desensitisation of the receptors, 4 cm strips of bladder tissue were dissected in half, and each tissue was set up in parallel. Identical conditions were followed for the two strips, with one acting as control in the presence of the vehicle. Specific drugs and concentrations are detailed in the results chapters.

2.1.6 Electrical field stimulation

Electrical Field Stimulation (EFS) was used to determine if urothelial contractions could be evoked from neuronal sources. Tissues were prepared and placed in Krebs solution at 37°C and gassed with 95% O₂ and 5% CO₂. In the organ bath, tissues were tied with cotton to a glass anchor, and the tissue was placed between two platinum-wire electrodes which ran alongside the length of the tissue. The upper end of the tissue was anchored to an isometric force transducer connected to a Powerlab system. After washing with warmed Krebs solution the tissues were allowed to equilibrate for an hour before commencing experiments.

Electrical field stimulation of the tissues was obtained using a Grass S48 stimulator via a *Med-Lab* Stimu-Splitter II (Figure 2-6). Measuring the electrical field stimulated contractions of the urothelium/lamina propria has the unique problem of the tissues displaying high rates of relatively strong spontaneous contractile activity. Stimulation

frequencies for this tissue were optimised in order to obtain contractions that were significantly neurogenic in origin, while also above the spontaneous contractile activity (amplitude of ~0.6-1.0 g).

The optimal stimulation parameters for urothelial/lamina propria strips were 5 second trains of pulses (0.1 - 0.5 ms pulse-width, 30 v) applied every 100 seconds. Up to four stimulation frequencies (5, 10, 15 and 20 Hz) were examined for each experiment and at the end of selected experiments tetrodotoxin (1 μ M) was used to confirm the contractions were neurogenic in origin. Responses to electrical stimulation at set frequencies were recorded before and after the addition of selected drugs. The amplitude of spontaneous contractions was calculated as the average amplitude for the contractions within 1 - 2 minutes prior to the electrical stimulation. The response was measured as the contractile amplitude above the average spontaneous contractile activity (Figure 2-7). Responses were compared in the absence and presence of various agonist and antagonists. Contractions in the presence of agonists were recorded at maximum response, usually within the first 2 - 4 minutes of incubation with the drug. Antagonists were left in the bath for 30 minutes to allow tissue equilibration tissues before contractions were measured.



Figure 2-6: The grass S48 Stimulator used to generate EFS signals. Pulses are supplied to 4 organ baths through the *Med-Lab Stimu-Splitter II* (above).

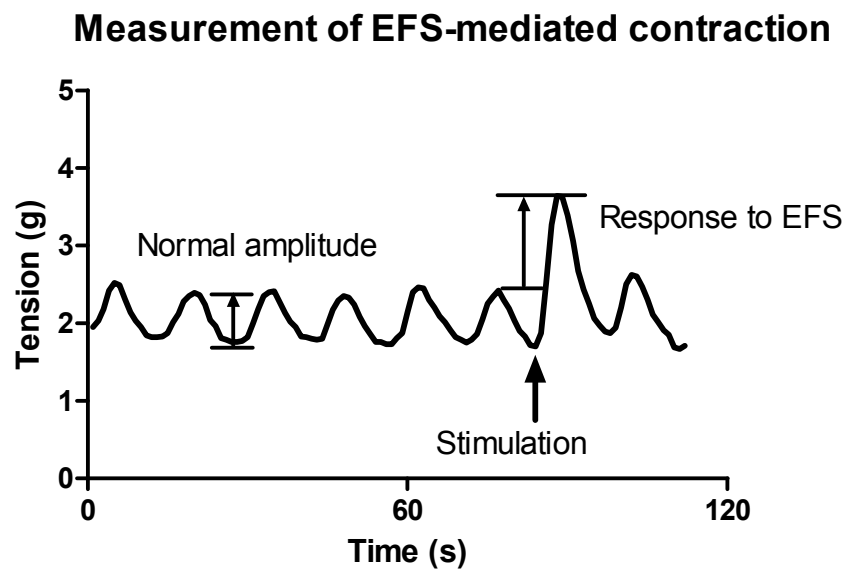


Figure 2-7: Demonstration of the methods used to calculate EFS-mediated contraction of the urothelium/lamina propria. This tissue exhibits spontaneous contractile activity. Total contractile amplitude was taken as contractions above the normal spontaneous amplitude.

2.1.7 *Analysis of data*

Data from each experiment were entered into Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). Measurements of frequency and amplitude for phasic activity were taken as an average value from 2 - 5 minutes prior to stimulation, and compared to measurements taken at the peak response after the addition of each drug unless otherwise stated. At peak responses, frequencies were taken over at least 2 minutes.

When possible, methods were developed so that tissues could be directly paired with the controls (ie: before and after drug in same tissue). For these experiments, values in the absence and presence of drugs were compared using Student's two-tailed paired *t*-test with $P < 0.05$ being taken as statistically significant, and the results presented as mean \pm SEM. Data from the experiment was exported to GraphPad Prism software v5 (GraphPad, San Diego, CA, USA) for statistical analysis. If tissues could not be paired directly an unpaired Student's *t*-test was applied for analysis of data. For experiments involving more than one treatment per control, one way analysis of variance (ANOVA) tests were performed followed by an appropriate post hoc test.

GraphPad Prism software v5 was used for the construction of concentration-response curves. After analysis of the individual curves, EC_{50} values were determined through a non-linear regression fit to a variable-slope concentration-response curve. GraphPad Prism uses a four-parameter logistic equation prism to calculate the curves. Each individual curve was designated a $-\log EC_{50}$ (pEC_{50}) value, and a mean (\pm SEM) of each separate tissue calculated. Experiments were designed to give a minimum of 6 recordings ($n \geq 6$) where possible prior to the calculations of these values. In some experiments a range of antagonist concentrations

was used and dissociation constants for these antagonists were determined. Schild regression analyses were constructed in Prism as detailed in past publications (Arunlakshana and Schild, 1959).

$$PK_B = \log(CR - 1) - \log[B]$$

Equation 1: pK_B = Dissociation constant. CR = Concentration ratio (ratio of the EC_{50} values). [B] = Concentration of the antagonist.

Data for some concentration-response curves were also calculated after normalising to 100%. The maximum contraction to an agonist was considered 100%, and GraphPad Prism software was used to extrapolate the interim values as a percentage of the maximum response. Concentration-response curves were constructed from this normalised data using a non-linear regression fitted with a sigmoidal variable slope function. pEC_{50} values were calculated for each individual curve, and the group data combined and averaged in Microsoft Excel or GraphPad Prism.

2.1.8 *Drugs and solutions*

Sources of drugs:

Carbamoylcholine chloride (carbachol), (R)- (-)-phenylephrine hydrochloride phenylephrine hydrochloride, α,β -methyleneadenosine 5'-triphosphate lithium (α,β -methylene ATP), tetrodotoxin (TTX), (\pm)-arterenol hydrochloride (noradrenaline), nifedipine, pyrilamine

maleate, guanethidine monosulphate, atropine sulphate, , protease inhibitor cocktail (P1860), phosphoramidon, histamine dihydrochloride, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), Nw-Nitro-L-Arginine (L-NNA), capsaicin, diethylamine NONOate sodium salt hydrate (DEANO), 18- β -glycyrrhetic acid, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), methocitramine hemihydrate, L-phenylephrine hydrochloride, sodium nitroprusside, phentolamine hydrochloride, and indomethacin, phosphoramidon, protease inhibitor cocktail (P1860), dimethyl sulfoxide, pirenzepine dichloride, , CL316243 disodium salt, (\pm)-Noradrenaline hydrogen tartrate, CGP20712A methanesulfonate salt, oxybutynin chloride, and prazosin hydrochloride were purchased from Sigma (St Louis, Missouri, USA).

Tamsulosin, mirabegron (YM-178), and solifenacin were gifted by Astellas, Leiderdorp, NL.

A61603 hydrobromide, BMY7378 dihydrochloride, BRL37344 sodium salt, CL316243 disodium salt, ICI118551 hydrochloride, (\pm)-propranolol hydrochloride, RS17053 hydrochloride, RS100329 hydrochloride, SR59230A, salbutamol sulphate, Clonidine hydrochloride and UK14,304 were purchased from Tocris (Ellisville, Missouri, USA).

The sources of other drugs, reagents and substances have been detailed where used.

Preparation of drugs

Indomethacin and nifedipine were dissolved in 100% ethanol. Capsaicin and the protease inhibitor cocktail were dissolved in 100% dimethyl sulfoxide (DMSO). The volume of drug added to the baths was kept to a minimum and the volume of ethanol and DMSO ($>0.3\%$

dilution) used as vehicles exerted no effect in control experiments. All other drugs were dissolved in distilled water and diluted in Krebs bicarbonate solution.

Krebs was composed of the following (name, molecular formula, [mM]): sodium chloride, NaCl, 118.4; glucose, C₆H₁₂O₆, 11.7; sodium hydrogen carbonate, NaHCO₃, 24.9; potassium chloride KCl, 4.7; magnesium sulphate, MgSO₄, 1.2; potassium dihydrogen monophosphate, KH₂PO₄, 1.2; and calcium chloride, CaCl₂, 1.9. Krebs solution was usually made up to 5 L solutions with distilled H₂O and stored at 4°C. Krebs was used within 24 hours from the initial dilution of salts.

2.1.9 Real-time quantitative PCR analysis

Total RNA extraction

Bladder tissues from 6 pigs were used to identify the transcriptomic expression of adrenoceptor RNA in the bladder urothelium/lamina propria). Strips of porcine bladder were immediately dissected at the abattoir and immersed in RNALater solution (Ambion). Within 1 hour from extraction, the bladder samples were placed at room temperature, finely dissected with scissors (cleaned with RNase away, Invitrogen) under magnification to remove all detrusor muscle fragments leaving ~0.25g of urothelium and lamina propria. A *Trizol plus* RNA purification kit (Invitrogen Cat No. 12183-555) was used for the urothelial RNA extraction. Dissected tissues were immediately immersed in Trizol and lysis of the tissue was achieved by passing the dissected fragments through a sterile pipette tip several times, then through sterile syringes of different gauges (19G and 23G). Following this procedure, the homogenate was left to incubate for 5 min at room temperature.

In order to facilitate the recovery of the aqueous phase of the PCRs which have been overlaid with mineral oil, 0.2 ml chloroform was added to the mixture. This suspension was rolled swiftly by hand for approximately 15 seconds to combine the solutions. Following 2 - 3 minutes incubation (25°C), the sample was centrifuged at 12,000 x g for 15 minutes at 4°C in an Allegra-64 centrifuge (Beckman Coulter). The colourless upper phase containing the RNA was collected and transferred into a sterile RNase free tube containing an equal volume of 70% ethanol, prepared from ultrapure RNase free water (Invitrogen) and pure ethanol. The sample was vortexed thoroughly for 30 seconds and placed in an RNA spin cartridge for further purification. Binding of the RNA and washing steps were undertaken following the manufacturer's instructions (Qiagen, 2010). The total RNA was then treated with DNase to digest any genomic DNA contamination. A mix containing: diethylpyrocarbonate water (40µl); RQ1 buffer (RNase-Free DNase, 20 µL) which is a preparation of deoxyribonuclease 1 that will degrade the single-stranded or double-stranded DNA in the mixture to produce oligonucleotides and thereby assist in maintaining high integrity RNA; and the redox reagent dithiothreitol (20µl of 0.1M) and deoxyribonuclease 1 (20µl of 2000 units/ml), which is a nuclease that cleaves DNA; was poured onto the RNA and left to incubate at 37°C for 15 minutes. RNA was eluted into 50 µl of RNase free pure water and maintained at minus 80°C to prevent decomposition. All experiments were performed within 3 months of the initial extraction and procedures were designed so that there was no unnecessary thawing of samples.

Purity, integrity and quantitative analysis of the RNA sample

A NanoDrop 1000 Spectrophotometer (Thermo Scientific) was used to measure the total RNA quantity and to assess the purity. The integrity of the RNA was also performed for all extractions by denaturing agarose gel electrophoresis and ethidium bromide staining (reagents from Qiagen, Hilden, Germany). The protocol used was adapted from Qiagen and is designed to provide enhanced sensitivity when analysing RNA fragments, as per manufacturer's recommendations (Qiagen, 2010).

A key feature of this protocol is that the concentrated RNA loading buffer, allows for a higher volume of RNA sample to be loaded onto the gel than conventional protocols (Sambrook, 1989). This procedure was performed with a 1.2% Formaldehyde Agarose (FA) Gel Electrophoresis prepared through 1.2g agarose, 10 mL 10x FA gel buffer (200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid), 50 mM sodium acetate, 10 mM EDTA, (PH stabilised at 7 using NaOH) which was then made up to 100 mL using RNase-free water. This mixture was then heated in order to melt the agarose and then cooled to 65°C in a water bath. 1.8mL of 37% (12.3 M) formaldehyde and 1 µL of 10 mg/mL ethidium bromide stock solution was added and mixed thoroughly. The mixture was then transferred to a gel support. Before running the gel the mixture was equilibrated in 1xFA gel running buffer consisting of 100 ml 10x FA gel buffer, 20 ml 37% formaldehyde, and 880ml RNase-free water, for 30 minutes.

All trays and tanks were initially washed in 70% ethanol, rinsed with DEPC water, washed with RNaseAway (Invitrogen) and left until dry. 8 µL total RNA was diluted to 100 ng/µL and mixed with a 5× RNA loading dye (16 µl saturated aqueous bromophenol blue solution,

80 µl 500 mM EDTA, pH 8.0, 720 µl 37% (12.3 M) formaldehyde, 2 ml 100% glycerol, 3.084 ml formamide, 4 ml 10 x FA gel buffer, RNase-free water to 10 ml). The RNA samples were loaded onto the wells cut into the gel, and following 5 minutes of incubation at 65°C they were electrophoresed at 90 Volts for 30 minutes. The gel was then visualised under an ultra violet transilluminator after ethidium bromide staining and photographed with a digital camera.

cDNA synthesis:

Synthesis of cDNA was performed using SuperScript III First-Strand Synthesis SuperMix from the qRT-PCR kit (Invitrogen). Initial incubation in a reaction mix [10 µL of 2X RT Enzyme Mix] containing superscript III and RNaseOUT was performed. Superscript III is a high-performance proprietary type of reverse transcriptase from Invitrogen Life Sciences, which has been optimised to synthesise first-strand cDNA from total RNA. RNaseOUT is a potent non-competitive inhibitor of ribonucleases. To this mixture, 2 µL of Reverse Transcriptase Enzyme Mix was added, which contained oligo(dT)₂₀ (2.5 µM), random hexamers (2.5 ng/µl), 10 mM MgCl₂, and dNTPs; 8µL of Eluted RNA (2 µg), and maintained at 25°C for 10 minutes, followed by a second incubation at 50°C for 30 minutes. The reaction was halted by bringing the mix to 85°C for 5 minutes. The overall cDNA was stored at -20°C and used within 3 months of freezing.

Quantitative real-time polymerase chain reaction:

An iQTM SYBR Green Super Mix (BioRad) was used to amplify the cDNA targeted genes in the Quantitative Real-Time Polymerase Chain Reaction (Q-PCR) process. This supermix

includes the SYBR green dye which binds to double stranded DNA as it is generated by PCR and allows the products to be quantified. Real-time PCR was performed with a Research Rotor-Gene 6000 real time thermocycler (Corbett-Qiagen). PCR volumes (containing 2 – 5 μ L cDNA) were placed in individual Corbett 100 μ L tubes on -20°C pre-cool metal tube carriers. All PCR tubes were placed in a 72 well rotor and held in the machine with a locking ring. Cyclic conditions were set which allowed specificity for each of the genes. 5 μ L of ADRB3 cDNA was used and amplified with the following conditions: 94°C 4 min (x1 cycle), cycle 2: 94°C 30 s, 51°C 15 s, 72°C 15 s (x40). All other genes were expressed with the conditions: 94°C 4 min (x1), Cycle 2, 94°C 30 s, 60°C 30 s, 72°C 30 s (x45). Prior to running the PCR, an amplification protocol was preset to obtain melt curve representations, ramping from 50 to 99°C with 1°C ramping every 5 seconds. The number of cycles required to the point that the specific PCR product is amplified in a linear way is referred to as the cycle threshold (CT). This can be used for quantitative analysis, where a single CT difference (Δ CT = 1) represents a two-fold difference in the amount of the specific target. Therefore, after each Q-PCR assay the CT value at linearity was determined for each gene. Realtime-PCR assays that demonstrated a non-specific product or primer-dimers at the end point were excluded from any further data collection. Expression levels of each target gene were expressed relative to the housekeeping gene, β -actin.

Primer selection:

Oligonucleotide primers were designed from the characterised pig genome (*Scrofa Domestica*). The primers (Table 1, Table 2) were designed specifically to bond to the targeted porcine genes and purchased from Geneworks (Thebarton, South Australia). The following porcine genes were identified within genebank through their codes:

ADRA1A, adrenergic alpha-1A receptor; ADRA1B, adrenergic alpha-1B receptor; ADRA1D, adrenergic alpha-1D receptor; ADRB1, adrenergic beta-1 receptor; ADRB2, adrenergic beta-2 receptor; ADRB3, adrenergic beta-3 receptor, ACTB, beta actin.

Gene	Forward Primer	Length	Tm	Amplicon size (bp)	Position	Number of exons	Primer Type
ADRA1A	CCATTGGGTCT TTCTTTCT	20	59.90	124	877	2	overlap
ADRA1B	GGTCATTCTGG TCATGTACTG	21	60.46	134	650	1	
ADRA1D	TTTCTTCGTCC TACCACTG	19	58.92	90	1091	2	span (17,000bp)
ADRB1	CCAACCTCTTC ATCATGTCC	20	60.42	144	277	1	
ADRB2	GATCCATGACA ACCTCATCC	20	60.20	117	890	1	
ADRB3	AGACTCCAGAC CATGACC	18	60.23	132	198	4	span (125bp)
ACTB	ATGGGCCAGAA GGACTC	17	60.59	144	138	6	span (132bp)

Table 1: Forward primers custom-designed for the identification of adrenoceptor genes

Gene	Reverse Primer	Length	Tm	Amplicon size (bp)	Position	Number of exons	Primer Type
ADRA1A	TTTAAACTCTT GACTGGAGCAC	22	60.83	124	1001	2	Overlap
ADRA1B	TCGTGAAAGTT CTTGGAGTG	20	60.20	134	784	1	
ADRA1D	GTTGAAGTAGC CCAGCC	17	59.91	90	1181	2	span (17,000bp)
ADRB1	CACAGTACGTC CACCGA	17	60.91	144	421	1	
ADRB2	GAAGGCCATCC TGAAATCTG	20	60.78	117	1007	1	
ADRB3	ACAGCTCGCAA CAAGTG	17	60.49	132	330	4	span (125bp)
ACTB	GCAGCTCGTTG TAGAAGG	18	60.38	144	282	6	span (132bp)

Table 2: Reverse primers custom-designed for the identification of adrenoceptor genes

Capillary electrophoresis:

The QIAxcel capillary gel electrophoresis system (Qiagen, Hilden, Germany) was used to provide a further visual identification of the primer binding affinity and sample quality. Post-PCR products samples were analysed using a high resolution gel cartridge with 2 μ L aliquots of the PCR product combined with a dilution buffer (Qiagen) to 10 μ L total volume. The QIAxcel system produces a digital gel image and an electropherogram for primer binding analysis.

Statistical analysis

Graphpad version 5 (PC) was used to collate and analyse all data collected. As each gene was performed with controls, a Student's two-tailed *t*-test was used to initially determine whether the ct values of the adrenoceptors were statistically significant from the housekeeping gene β -actin for each cDNA sample (with $P < 0.05$ being significant, and groups with a SD < 0.8 being acceptable).

Chapter 3

OPTIMISATION OF TECHNIQUES

3.1.1 *Selection of an animal model*

Porcine bladder was used in this study as a suitable and effective model for research related to human bladders (Parsons et al., 2012). This tissue is structurally similar to the human bladder, and shares similar physiology and pharmacology (Sibley, 1984, Crowe and Burnstock, 1989, Templeman et al., 2003, Buckner et al., 2000, Kumar et al., 2004). In addition, diseases prevalent in human bladders, such as detrusor overactivity, have been demonstrated in pig models (Jorgensen et al., 1983, Sibley, 1985, Speakman et al., 1987, Chapple and Smith, 1994).

Large White-Landrace-Duroc crossbred pig bladders from a local abattoir were used in this study. It was important to identify a suitable size, age and health from this breed. On request, the abattoir did not supply any tissues where the pigs were found to be infected with disease, and all pigs used in this study were fit for human consumption. In addition, prior to the initial dissection of the tissue, each pig bladder used in this study was visually checked for pus, blood or infection. Bladders were used within 24 hours of removal from the pigs. As an additional measure of pig health, an *ABL 80 Flex Co-ox* radiometer was transported to the abattoir where it was set up to receive the fresh blood immediately from the pig, and the analysis for each sample was commenced within 1-5 minutes of the blood being removed from the pig (n = 6). Each pig's blood analysis was consistent with healthy reference values.

Some studies such as EFS and a range of organ-bath experiments required a single dose of an antagonist. The chosen concentration was selected as the highest possible concentration which remained selective for the individual receptor. Tables of affinity values were compiled from the literature for the antagonists used in the study (Table 3, Table 4, Table 5).

Muscarinic receptor-selective antagonists

Antagonist	M ₁		M ₂		M ₃		M ₄		M ₅	
	mean	range	mean	range	mean	range	mean	range	mean	range
4-DAMP	9.28	9.24-9.40	8.30	8.14-8.40	9.28	9.10-10.60	8.93	8.43-9.14	8.98	8.3–9.25
Darifenacin	7.82	7.50-8.15	7.17	6.94-7.40	8.64	8.01-9.12	7.65	7.34-7.90	7.99	7.61–8.26
Methoctramine	7.12	6.55-7.50	8.05	7.56-8.7	6.67	6.05-6.80	7.31	6.85-7.6	6.65	6.3 – 7.00
Pirenzepine	8.15	8.04-8.20	6.47	6.28-6.65	6.89	6.40-7.44	7.39	6.98-7.76	6.95	6.40-7.20
Tolterodine*	7.79	±0.62	7.99	±0.72	8.10		8.58	±0.90	8.62	±0.57
Solifenacin*	7.08	±0.55	7.03	±0.70	7.72	±0.63	7.73	±0.92	7.46	±0.57
Oxybutynin*	8.62	±0.73	8.17	±0.90	9.17	8.2-9.17	8.90	±0.93	7.96	±0.11

Table 3: Published values of antagonist affinities for M₁ – M₅ subtypes. Data presented as mean ± SEM (Chess-Williams et al., 2001; Dorje et al., 1991; Eglen et al., 2001; Eglen et al., 2000; Hegde et al., 1997; Moriya et al., 1999; Sellers et al., 2000b; Wallis et al., 1999; Wang et al., 1995; Watson et al., 1999). The mean affinity value given in the table was derived from the quoted affinity estimates (pK_B or PK_i), the range reflects both the lowest and highest affinity estimate at a given receptor subtype from the literature. Functional data is limited for some drugs, and those denoted with an “*” are from radioligand binding data to human receptors expressed in CHO cells and are sourced from (Wuest et al., 2006, Mansfield et al., 2009, Mansfield et al., 2005, Nilvebrant et al., 1997, Ohtake et al., 2007).

α -adrenoceptor antagonists

Antagonist	α_{1A}		α_{1B}		α_{1D}	
	mean	range	mean	range	mean	range
BMY7378	6.5	6.1- 7.1	6.9	6.2- 7.4	8.9	8.2- 10.5
Prazosin	9.3	8.8– 9.9	9.6	9.0- 10.0	9.6	9.4- 10.2
RS100329	9.6	9.6– 9.7	7.8	7.5- 8.0	7.9	7.9- 8.0
RS17053	9.4	9.3- 9.5	7.6	7.2- 8.0	7.4	7.1- 7.8

Table 4: Published values of antagonist affinities for α -adrenoceptor subtypes from both functional and binding studies (Michel & Insel, 1994; Burt, Chapple & Marshall, 1995; Goetz et al., 1995; Kenny, Chalmers, Philpott & Naylor, 1995; Buckner et al., 1996; Marshall, Burt, Green, Hussain & Chapple, 1996; Ford et al., 1997; Noble et al., 1997; Langer, 1999; Williams et al., 1999; Mackenzie, Daly, Pediani & McGrath, 2000, Bagot, K, Thesis 2005). Data presented as mean \pm SEM.

β -adrenoceptor antagonists

Antagonist	β_1		β_2		β_3	
	mean	range	mean	range	mean	range
ICI118551	6.81	6.15-7.38	8.68	8.34-9.22	5.45	5.1-5.8
CGP20712A	8.8	8.48-9.30	5.29	4.4-5.82	< 4	< 4
SR59230A	6.39	(pIC ₅₀)	6.19	(pIC ₅₀)	8.7	8.6-8.8
Propanolol	8.76	8.4-9.0	8.83	8.13-9.20	6.14	5.8-7.02

Table 5: Published values of antagonist affinities for β_1 - β_3 subtypes (Bylund and Larry, 2009, Bylund et al., 2007b, Bylund et al., 2007c, Bylund et al., 2007a, Louis et al., 1999, Bylund et al., 1994, Yamanishi et al., 2002b, Candelore et al., 1999, Alexander et al., 2011). The mean affinity value given in the table was derived from the quoted affinity estimates (pK_B or PK_i), the range reflects both the lowest and highest affinity estimate at a given receptor subtype from the literature. Data presented as mean \pm SEM.

3.1.2 *Real-time quantitative PCR analysis*

Polymerase Chain Reaction is the optimal method used in laboratories to amplify strands of actively transcribed DNA. Current technologies utilise Real-Time PCR which allows accurate rates of DNA amplification. The enzyme DNA polymerase commences amplification and replication of activated DNA strands in live cells. In the laboratory, for PCR to replicate this process, the enzyme requires specific primers to be bound to the DNA. These are called the forward primers (5'). Replication also occurs on the complimentary strand of DNA, where the reverse primer (3') binds. At suitable conditions, primers can be designed for specific genes and will bind effectively to DNA at the forward 5' end and the reverse 3' end of a targeted gene.

Expressed genes in a tissue require encoding from messenger RNA (mRNA). Rather than amplify an entire DNA strand, specific genes can be identified through extracting this mRNA from a tissue. The mRNA is then used to synthesise a section of complementary DNA (cDNA), which can be amplified in the laboratory using PCR. Each PCR cycle exponentially amplifies a targeted region of cDNA. This non-linear increase in the quantities of DNA after each cycle means that conventional methods of PCR are unable to quantify the relative expressions of each gene (Kainz, 2000, Sambrook, 1989). New technologies have been developed which not only allow basic quantification, but which can also indicate the ratio of expression between genes in each cDNA sample (Klein, 2002). This contemporary method of Real-Time PCR allows the researcher to identify at which PCR cycle the targeted gene becomes amplified in a linear way, which is referred to as the Cycle Threshold (CT) value (Ginzinger, 2002). On the logarithmic linear scale, each increase in the CT value is indicative of a doubling in the concentration of the targeted gene. In processing this data, an

absolute PCR analysis is completed, which allows for the determination of the abundance of a particular gene in respect to others in the same sample of cDNA.

The expression and relative abundance of the adrenoceptors in the pig urothelium is currently unknown. Analysis of the porcine receptors through mRNA analysis was focussed on ascertaining which genes were present in this tissue, and detecting their abundance relative to the housekeeping gene β -actin using Real-Time Quantitative PCR (Q-PCR) analysis. Special attention was drawn to select an appropriate housekeeping gene for porcine tissue, and to determine accordingly, the levels of expression of the alpha and beta adrenoceptors following the optimisation of all PCR parameters for each gene of interest.

3.1.3 Optimal conditions for the extraction and assessment of total RNA from porcine bladder tissue.

Extraction of total RNA from bladder urothelium and lamina propria sections:

The process outlined in the methods section of this thesis (Real-Time Quantitative PCR Analysis) was analysed to determine the quantity, purity, integrity and size distribution of the RNA samples extracted.

NanoDrop 1000 Spectrophotometer (Thermo Scientific) was used to measure the total RNA quantity and to assess the purity. RNA concentrations and 260/280 ratios are tabulated in Table 6.

Table 6: Nanodrop recordings for each RNA sample

RNA Sample	A260	A280	Ratio 260/280	Ratio 260/230	ng/ μ L
1	15.06	7.327	2.06	1.41	602.38
2	17.479	8.316	2.10	2.18	699.17
3	26.253	12.646	2.08	2.22	1050.11
4	15.504	7.408	2.09	2.20	620.16
5	20.577	9.818	2.10	2.21	823.10
6	14.747	6.974	2.11	2.09	589.88

To further demonstrate the quality of the extracted RNA, a gel electrophoresis was run. Across all samples of bladder urothelium and lamina propria (1 - 6), the 28s band was higher in intensity compared to the 18s band (Figure 3-1). This demonstrates a good integrity of the large molecules of RNA with no significant degradation. All bands appear sharp and none have significantly started to smear towards the smaller sized RNA. This demonstrates that the procedure used to extract RNA from the bladder was effective in isolating high-integrity samples. The RNA was taken from the vials stored at minus 80, and as a result, the bands in Figure 3-1 also demonstrate that the sample has not undergone any major degradation during the freeze-thaw processing prior to experimentation.

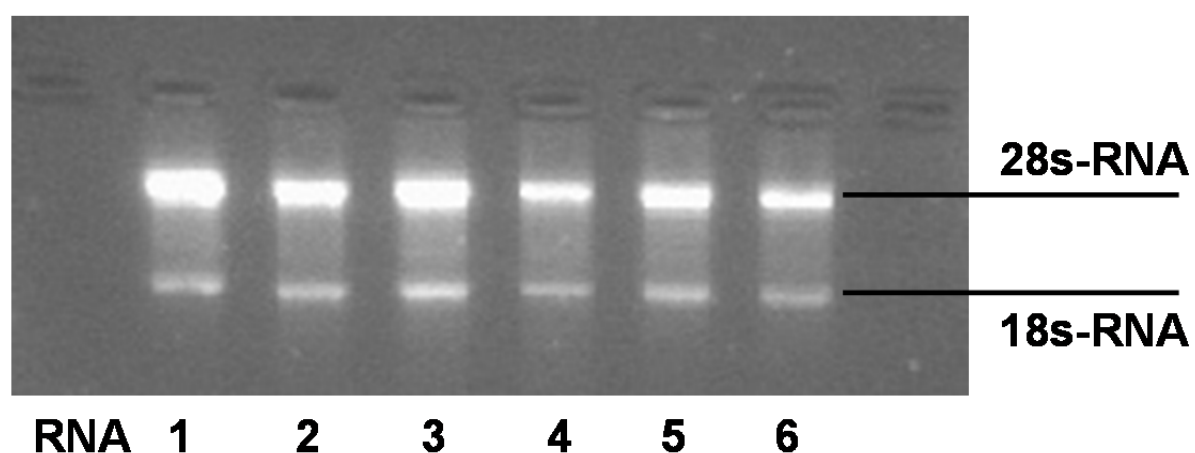


Figure 3-1: RNA integrity shown in a 2% formaldehyde agarose gel. Across all 6 samples the RNA extracted from the urothelium and lamina propria demonstrated high intensities of both the 28s band and the 18s band. The clear visibility and clarity of both bands demonstrates a good integrity of the RNA, with minimal degradation.

Selection of a housekeeping gene for porcine adrenoceptors

In order to determine the expression levels of adrenoceptors in porcine bladder urothelium/lamina propria, it is necessary for the QPCR analysis and interpretation of results to compare the real time PCR cycle thresholds of candidate genes with a common, known gene. It is thus important to determine an optimum reference gene which can be used to normalise variations within individual adrenoceptor genes. Quantification of the mRNA of the target and the housekeeping gene in a sample ensures that changes in transcript levels will influence both genes equally. Identification of a viable reference gene was therefore carried out to ultimately obtain a stable, equivalent expression in all porcine bladder RNA samples. Two genes, 18S, and β -actin are commonly used for normalization purposes in many expression studies. However, transcript levels of these genes are known to vary between different types of animal, tissue (normal and pathological samples) and under different treatment conditions (drugs and chemicals). Analysis of the PCR product of these genes has been undertaken to assist in identifying the correct gene for use in porcine bladder tissue.

18S RNA primer analysis

A common housekeeping gene for QPCR analysis is 18S ribosomal RNA (Derks et al., 2008). This ribosomal subunit is consistently used as a standard when identifying levels of expression and the integrity of samples. In comparison to other housekeeping genes, these ribosomal 18S subunits show the most consistent steady-state expression levels (Sturzenbaum and Kille, 2001). Realtime PCR of 18S across all cDNA samples is shown in Figure 3-2

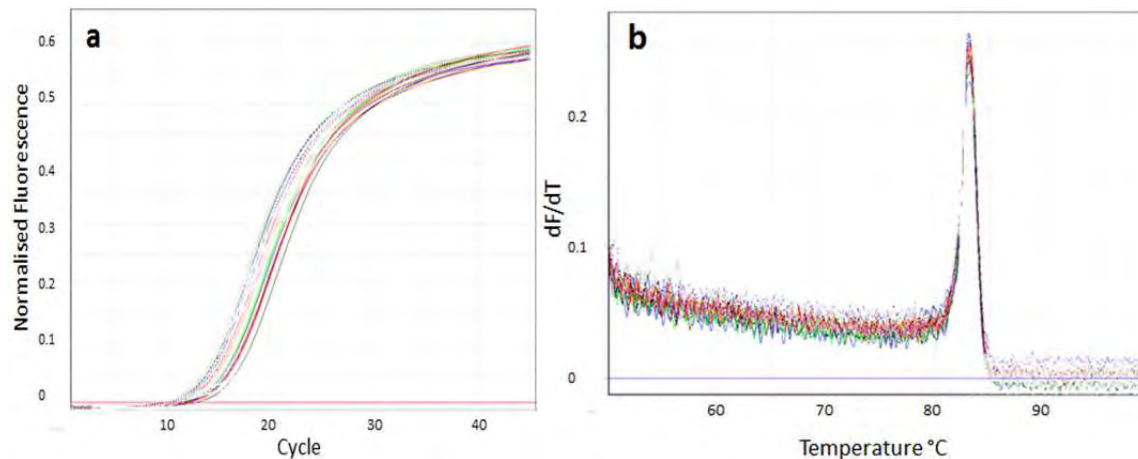


Figure 3-2: (a) PCR cycling graph showing evidence for a high expression of 18S RNA in urothelial tissues (n = 6 tissues, each run in duplicate). (b) Melt curve analysis demonstrating homogenous PCR size amplification of the 18S gene.

β-actin primer analysis

β-actin is one of the major components in cytoplasmic microfilaments in eukaryotic cells, and plays important roles in a range of cell cytoskeleton functions. Functional processes involving β-actin include phagocytosis, cytoplasmic streaming, cell division and alterations to cell shape (Romans et al., 1985, Kusakabe, 1997). Every organism possesses a range (usually three to four) of actin isoforms and the total actin content can vary with cell conditions, pathologies and muscle type (Drew and Murphy, 1997). Nonetheless, actin is widely used as a housekeeping gene, and β-actin has been used in a range of different QPCR studies where it has generally exhibited good housekeeping gene characteristics (Raff et al., 1997). Realtime PCR of β-actin across all cDNA samples is shown in Figure 3-3. End-point PCR have been run through agarose gel electrophoresis and a QIAxcel to rule out the presence of any additional discrete extra bands (Figure 3-4, Figure 3-5).

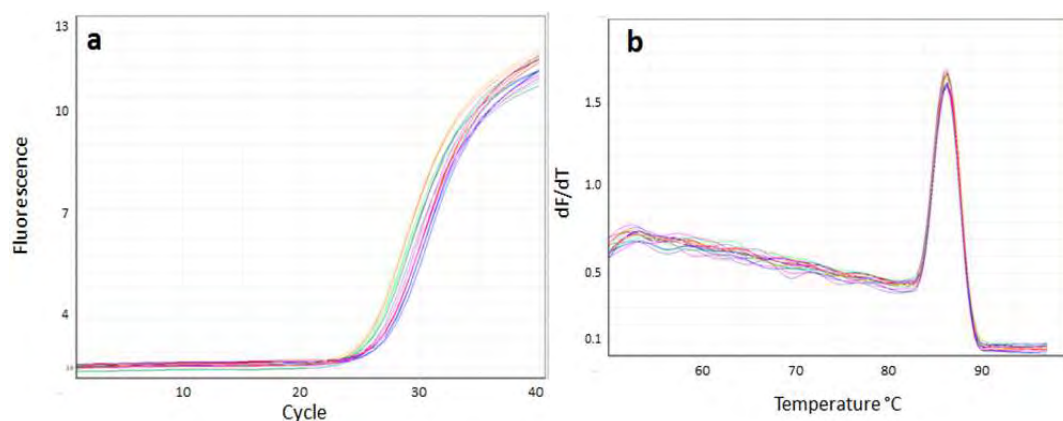


Figure 3-3: (a) PCR cycling graph showing the exponential increases in fluorescence for the β -actin gene. This gene was highly prevalent in all samples of urothelial tissues (n = 6 tissues, each run in duplicate). (b) Melt curve analysis demonstrated specific amplicons of the same size.

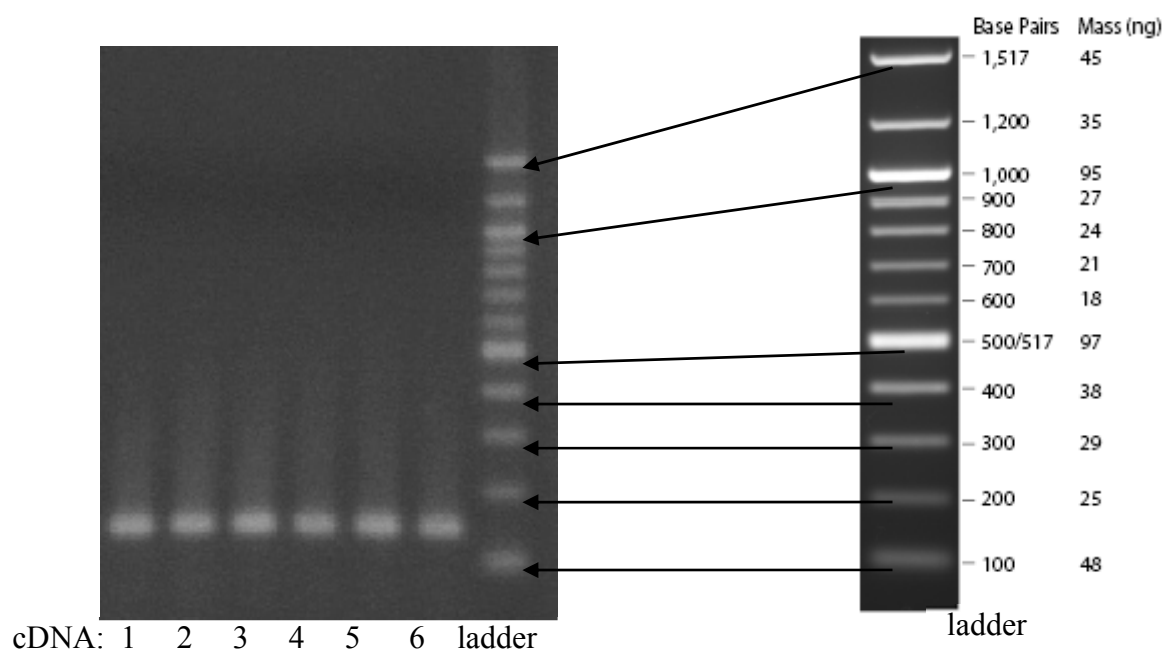


Figure 3-4: Gel showing the fragment sizes of the 6 cDNA samples after binding with the β -actin gene (amplicon size: 144 bp).

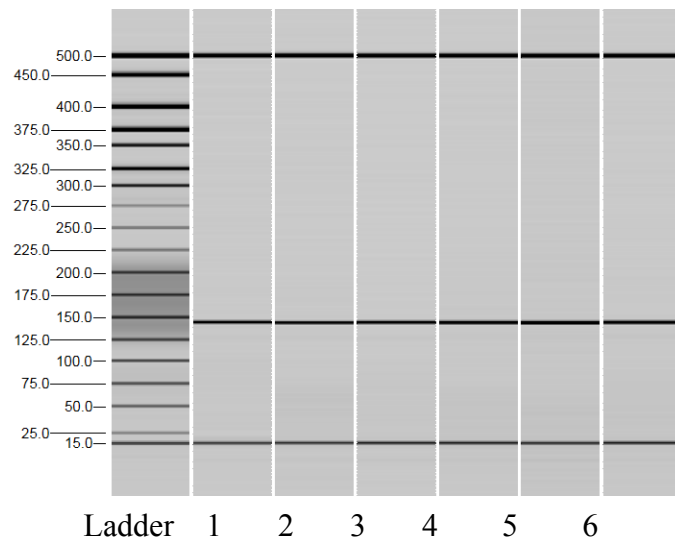


Figure 3-5: Digital electropherograms from a QIAxcel system of the 6 PCR fragments of β -actin from urothelial/lamina propria cDNA.

Comparison of suitable housekeeping genes between β -actin and 18s RNA genes for QPCR analysis of porcine samples:

18S RNA is highly prevalent across all tissues as the average cycle threshold was 11.18. β -actin, however, appeared to commence an exponential increase later than the 18S gene, with its average cycle threshold at 21.15. This value was a closer match to the targeted values. In addition, the primers designed for β -actin showed a high affinity with the cDNA during PCR. As a result, β -actin was chosen as a suitable housekeeping gene with which to compare the adrenoceptor results.

3.1.4 *Optimising conditions for real-time quantitative polymerase chain reaction of porcine adrenoceptors*

Preliminary experiments were performed on each targeted gene to identify the optimal conditions for the expression. Table 7 identifies individual optimal PCR conditions for each gene expression.

Table 7: Optimal PCR parameters found for each individual gene

Gene	Preheat		Denaturing		Annealing		Extension		Cycles
ADRA1A	94°C	4min	95°C	30sec	60°C	30sec	72°C	30sec	45
ADRA1B	94°C	4min	95°C	30sec	60°C	30sec	72°C	30sec	45
ADRA1D	94°C	4min	95°C	30sec	60°C	30sec	72°C	30sec	45
ADRB1	94°C	4min	95°C	30sec	60°C	30sec	72°C	30sec	45
ADRB2	94°C	4min	95°C	30sec	60°C	15sec	72°C	15sec	45
ADRB3	94°C	4min	95°C	30sec	51°C	15sec	72°C	15sec	40
ACTB	94°C	4min	95°C	30sec	60°C	30sec	72°C	30sec	45
18S RNA	94°C	4min	95°C	30sec	60°C	30sec	72°C	30sec	45

3.1.5 *Determination of an optimal β_3 -adrenoceptor primer set quantitative real time PCR using porcine cDNA*

Throughout initial PCR parameters, the custom designed β_3 -adrenoceptor primer sequence exhibited poor binding to the gene with unsatisfactory PCR results. However, other sets of primers demonstrated the β_3 -adrenoceptor gene in porcine urothelium/lamina propria mRNA. Further optimisation was completed on this tissue such as altering the dilution factor of cDNA templates; the volume of cDNA in the PCR tubes; the annealing and extension PCR temperatures; and more specific sets of primers. These methods contributed to obtaining the optimal real-time PCR parameters for each gene. In addition, alternate primers were also

assessed on this gene. Although the β_3 -adrenoceptor mRNA expression has not yet been successfully completed in the porcine urothelium and lamina propria, a recent publication has determined the levels of β -adrenoceptors in human bladders (Otsuka et al., 2008). The β_3 oligonucleotide sequence in porcine is not fully known yet the sequence of the gene in human urinary bladder is roughly 98% similar. It was hypothesised that the ADRB3 oligonucleotide primers designed through the ADRB3 human sequence (GeneBank accession Number NM_000025) may bind with similar affinity to the porcine gene and provide satisfactory PCR amplification (Table 8). However, upon analysis of the PCR recordings, and the post-PCR products (Figure 3-6) the use of this primer was deemed unsuitable in porcine bladder analysis of β_3 -adrenoceptor gene expression by QPCR.

The customised porcine β_3 -adrenoceptor primers, devised specifically using the porcine genome (Table 8), were able to provide a reliable PCR product following various optimisation procedures. Agarose gel electrophoresis demonstrated an increased affinity for the primers at lower annealing temperatures and annealing time. The final optimised settings of both time and annealing temperature were 51°C and 15 seconds annealing respectively. Extension was set at 72°C for 15 seconds. Optimised final PCR settings provided expected sized β_3 gene amplification using β_3 -specific primers (Figure 3-7, nonselective binding is indicated with an arrow).

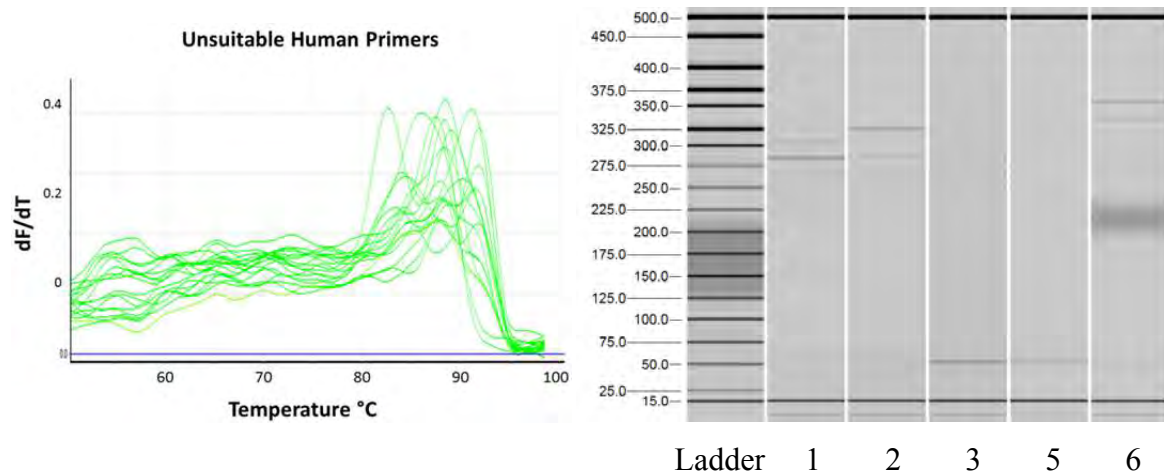


Figure 3-6: Melt curve and electropherograms:

[Left]: Melt curve analysis of the human β_3 -adrenoceptor expression in porcine tissue. The graph shows unspecific melt curve profiling and demonstrates a very poor affinity for primers at end product PCR. These primers were deemed unsuitable for the purpose of this study.

[Right]: Digital electropherograms from QIAxcel system demonstrating non-specific expected size of β_3 -adrenoceptor amplicons post PCR, demonstrating a poor affinity of the human β_3 -primers screened (Otsuka et al., 2008) on porcine urothelial/lamina propria cDNA.

Gene	Forward Primer	Reverse Primer
ADRB3 (Porcine)	5' AGACTCCAGACCATGACC 3'	5' ACAGCTCGCAACAAGTG 3'
ADRB3 (Human)	5' GCTCCGTGGCCTCACGAGAA 3'	5' CCCAACGGCCAGTGGCCAGTCAGCG 3'

Table 8: Two types of primers used for analysis of the porcine β_3 -adrenoceptor. ADRB3 (Human, GeneBank accession number NM_000025) was ineffective at providing a consistent PCR product with a high affinity.

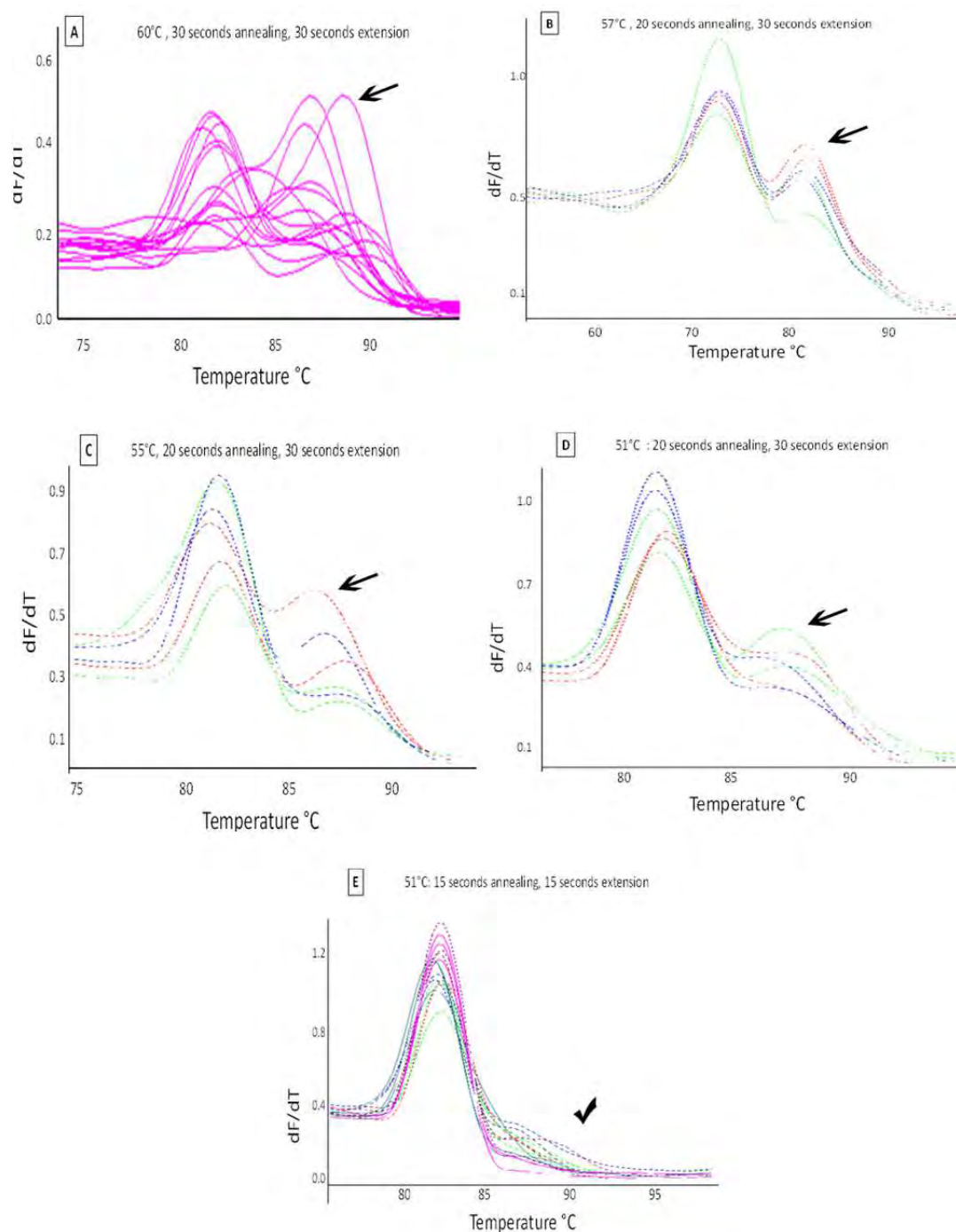


Figure 3-7: Melt curves for each $\beta 3$ gene showing the affinity of primers for the gene product. Arrows point on the product of non-desired non-specific amplification.

- A: 94°C denat / 30 sec; 60°C annealing / 30 sec; 72°C extension / 30 sec.
 B: 94°C denat / 30 sec; 57°C annealing / 20 sec; 72°C extension / 30 sec.
 C: 94°C denat / 30 sec; 55°C annealing / 20 sec; 72°C extension / 30 sec.
 D: 94°C denat / 30 sec; 51°C annealing / 20 sec; 72°C extension / 30 sec.
 E: 94°C denat / 30 sec; 51°C annealing / 15 sec; 72°C extension / 15 sec.

E represents the optimal conditions

Chapter 4

Urothelial/lamina propria spontaneous activity and the role of M3 muscarinic receptors in mediating rate responses to stretch and carbachol

The content in the following chapter is presented as the author's copy of the published document, prior to the reviewer and publisher's requested alterations. The final version of this manuscript can be accessed from the relevant peer-reviewed journal. Formatting and terminology changes have been made to maintain consistency throughout this thesis

All data presented in this manuscript is the sole work of C. Moro.

Published manuscript

Moro C, Uchiyama J, Chess-Williams R (2011). Urothelial/Lamina propria spontaneous activity and the role of M3 muscarinic receptors in mediating rate responses to stretch and carbachol. *Urology* 78(6): 1442.e9-15.

Published abstracts and conference presentations arising from this chapter:

Moro C, Milligan C, Leeds C, Chess-Williams R (2009). Spontaneous contractile activity of the urothelium is increased by muscarinic and purinergic receptor stimulation. *Neurology and Urodynamics* 28(7): 867-868. San Francisco, USA.

Moro C, Chess-Williams R (2009). Autonomic control of urothelial spontaneous contractile activity. *Proceedings of the 43rd Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists Scientific Meeting* 43.

Moro C, Chess Williams R (2009). Altering the affects of urothelial spontaneous contractions. *Proceedings of the 1st National Symposium on Advances in Urogenital and Gut Research* 1(1).

Moro C, Chess-Williams R (2010). Characterisation of the muscarinic receptor subtype regulating urothelial spontaneous contractile activity. *Proceedings of the 44th Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists Scientific Meeting* 44. Available at: <http://ascept.e proceedings.com.au/papers/p105.pdf>

Moro C, Chess Williams R (2010). Carbachol-induced increases in spontaneous contractile activity are mediated through the M3-muscarinic receptor. *Proceedings of the 2nd National Symposium on Advances in Urogenital and Gut Research* 1(1).

4.1 ABSTRACT

Objectives: To investigate the effects of tissue stretch and muscarinic receptor stimulation on the spontaneous activity of the urothelium/lamina propria and identify the specific receptor subtype mediating these responses.

Methods: Isolated strips of porcine urothelium with lamina propria were set up for *in vitro* recording of contractile activity. Muscarinic receptor subtype selective antagonists were used to identify the receptors influencing the contractile rate responses to stretch and stimulation with carbachol.

Results: Isolated strips of urothelium with lamina propria developed spontaneous contractions ($3.72 \text{ cycles min}^{-1}$) that were unaffected by tetrodotoxin, L-NNA or indomethacin. Carbachol ($1 \text{ }\mu\text{M}$) increased the spontaneous contractile rate of these tissue strips by $122 \pm 27\%$ ($P < 0.001$). These responses were significantly depressed in the presence of the M_3 -selective muscarinic antagonist 4-DAMP ($10 - 30 \text{ nM}$), but not affected by the M_1 -selective antagonist pirenzepine ($30 - 100 \text{ nM}$) or the M_2 -selective antagonist methoctramine ($0.1 - 1 \text{ }\mu\text{M}$). Stretching of the tissue also caused an increase in spontaneous contractile rate and these responses were abolished by atropine ($1 \text{ }\mu\text{M}$) and low concentrations of 4-DAMP (10 nM). Darifenacin, oxybutynin, tolterodine and solifenacin ($1 \text{ }\mu\text{M}$) all significantly depressed frequency responses to carbachol ($1 \text{ }\mu\text{M}$).

Conclusions: The urothelium with the lamina propria exhibits a spontaneous contractile activity which is increased during stretch. The mechanism appears to involve endogenous acetylcholine release acting on M_3 muscarinic receptors. Anticholinergic drugs used clinically depress responses of these tissues and this mechanism may represent an additional site of action for these drugs in the treatment of bladder overactivity.

4.2 INTRODUCTION

Detrusor overactivity is a common condition affecting up to 17% of the population (Milsom et al., 2001). In this condition spontaneous bladder contractions occur during the filling stage of the micturition cycle, but the mechanisms involved are unknown. Local mechanisms appear to be involved since spontaneous contractile activity of isolated tissues has been observed (Jiang et al., 2005). In recent years the urothelium and the underlying lamina propria have been recognized as important regulators of bladder activity, releasing factors that modulate detrusor contraction (Hawthorn et al., 2000, Templeman et al., 2002b) and sensory nerve activity (Cockayne et al., 2000). Furthermore, these roles may be clinically relevant since the inhibitory effect these tissues have on detrusor contraction is depressed in the neurogenic overactive bladder (Chess-Williams, 2009), while non-neuronal ATP release from this tissue is enhanced in the bladders of patients with painful bladder (sensory) syndrome (Kumar et al., 2007). Thus the urothelium and lamina propria are known to play important roles in maintaining normal bladder function and are associated with the development of pathological states.

In addition to releasing ATP and other mediators, the urothelium/lamina propria of the pig bladder (Sadananda et al., 2008) and the lamina propria of the rabbit urethra (Mattiasson et al., 1985a, Zygmunt et al., 1993) display contractile properties and have been shown to contract in response to a number of agonists. It is presently unclear which specific cell types mediate contraction, but it has been suggested that interstitial cells may be involved (Sadananda et al., 2008). In addition, it has been reported that spontaneous contractions of intact bladder strips may be linked to the urothelium/lamina propria (Akino et al., 2008). Also, these tissues from the dome of the pig bladder have been shown to develop spontaneous

phasic contractile activity (Moro and Chess Williams, 2010). Again it is unclear which cell types might act to initiate this spontaneous activity, but interstitial cells in the lamina propria have been shown to develop spontaneous depolarisations and calcium transients, which may suggest a possible pacemaker role (Kanai et al., 2007). The present study has examined the contractile activity of isolated strips of urothelium/lamina propria with the aim of identifying the muscarinic receptor subtype responsible for regulating the frequency of spontaneous contractions and to investigate the effects of tissue stretch on the spontaneous rate of contraction.

4.3 MATERIALS AND METHODS

Fresh bladders from Large-White Landrace pigs (6 months old, 80 Kg) were obtained from a local abattoir and immediately immersed in cold Krebs-bicarbonate solution (composition in mM: NaCl 188.4, NaHCO₃ 24.9, CaCl₂ 1.9, MgSO₄ 1.15, KCl 4.7, KH₂PO₄ 1.15 and D-glucose 11.7). The bladders were opened longitudinally and full thickness strips of anterior wall from the dome region were removed. From these tissues, strips of urothelium including the lamina propria were prepared (2 cm x 5 mm). For smooth muscle experiments detrusor strips were examined after removal of the urothelium and lamina propria. All tissues were immersed in Krebs-bicarbonate solution, maintained at 37°C and gassed with 5% CO₂ in oxygen.

The tissues were attached to isometric force transducers (ADInstruments MCT050/D) and tension recorded with a Powerlab system (ADInstruments, Castle Hill, Australia) using Labchart v7 software. After setting to a baseline tension of 2g the tissues were washed several times with fresh Krebs solution and allowed to equilibrate for 45 minutes before

starting drug additions. All antagonists were left to incubate for at least 20 minutes in the organ baths prior to further agonist additions. In stretch experiments, tissues (with or without the selective antagonist) were stretched by increasing the length of the tissue by 75% and the spontaneous contractile frequency was recorded before and after stretching.

Measurements of frequency, amplitude and baseline tension were taken at the peak response after the addition of each drug or stretching, as detailed in sections 2.1.1 - 2.1.4 of this thesis. The frequency of contractions was expressed as the number of phasic waves per minute and the amplitude as grams tension. The baseline tension was taken as the lowest point of the spontaneous phasic contractions. Mean (\pm SEM) values in the absence and presence of drugs were compared using Student's two-tailed paired *t*-test with $P < 0.05$ being taken as statistically significant. Prism software (GraphPad, San Diego, CA, USA) was used for statistical analysis of data.

Acetylcholine chloride (ACh), carbamoylcholine chloride (carbachol), α,β -methyleneATP lithium ($\alpha\beta$ mATP), tetrodotoxin (TTX), atropine sulphate, N^w-nitro-L-arginine (L-NNA), pirenzepine dihydrochloride, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), 1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP), methoctramine hemihydrate, indomethacin and oxybutynin chloride were purchased from Sigma (St Louis, MO, USA). Darifenacin hydrobromide, solifenacin succinate and tolterodine tartrate were gifts from Astellas. Indomethacin was dissolved in 100% ethanol and control experiments were performed using vehicle alone. All other drugs were dissolved in distilled water and diluted in Krebs-bicarbonate solution.

4.4 RESULTS

4.4.1 *Spontaneous phasic contractions*

The preparations of urothelium and lamina propria exhibited spontaneous contractions within 10 minutes of being placed in the organ bath. This regular phasic activity occurred at a spontaneous contractile frequency of 3.72 ± 0.12 cycles min^{-1} with an amplitude of $0.71 \pm 0.05\text{g}$ ($n = 53$, Figure 4-1). The frequency and amplitude of spontaneous contractions, and the baseline tension of tissues, were not affected by either tetrodotoxin ($1 \mu\text{M}$, $n = 12$), demonstrating a lack of neuronal input, indomethacin ($5 \mu\text{M}$, $n = 7$), indicating a lack of prostanoid activity, L-NNA ($100 \mu\text{M}$, $n = 10$) indicating no significant input from nitric oxide, or the nicotinic receptor agonist DMPP ($100 \mu\text{M}$, $n = 8$). In contrast to the urothelial/lamina propria activity, only 25% of detrusor smooth muscle strips ($n = 28$) set up under identical conditions, developed spontaneous activity and the spontaneous rate of the active tissues (1.57 ± 0.20 cycles min^{-1}) was significantly lower than that of the urothelium/lamina propria strips ($P < 0.001$).

4.4.2 *Muscarinic receptor stimulation*

The frequency of spontaneous urothelium/lamina propria contractions was not affected by atropine ($1\text{-}30 \mu\text{M}$, $n = 16$). Addition of acetylcholine ($1 \mu\text{M}$) however, increased the frequency by $39 \pm 14\%$ ($n = 8$, $P < 0.05$). Carbachol ($1 \mu\text{M}$) produced a greater increase in frequency than acetylcholine ($1 \mu\text{M}$), increasing the spontaneous rate by $122 \pm 27\%$ (Figure 4-1). Carbachol ($1 \mu\text{M}$) also increased the baseline tension by $152 \pm 13\%$ ($P < 0.001$, $n = 27$) and the amplitude of spontaneous phasic contractions was also reduced by $47 \pm 4\%$, ($P <$

0.001, $n = 27$). Increases in frequency induced by carbachol (1 μM) were not affected by the addition of indomethacin (5 μM) or L-NNA (100 μM).

To identify which muscarinic receptor subtype mediated the increase in urothelium/lamina propria spontaneous rate, responses to carbachol (1 μM) were obtained in the presence of receptor-selective antagonists (Figure 4-2, Table 3 in Chapter 3 of this Thesis). The M_3 -selective antagonist 4-DAMP (10 nM) had the greatest effect on responses, more than halving the carbachol-induced (1 μM) increase in frequency from $83 \pm 21\%$ to $35 \pm 8\%$ ($P < 0.01$ $n = 12$). A higher concentration of 4-DAMP (30 nM) further depressed responses to carbachol (1 μM , $18 \pm 4\%$, $n = 8$). The M_1 -receptor selective antagonist pirenzepine at concentrations up to 100 nM had no significant affect on the responses to carbachol ($n = 12$). At a concentration of 100 nM ($n = 9$) the M_2 -receptor selective antagonist methoctramine had no effects on the frequency or tension responses to carbachol, but at a higher concentration (1 μM , $n = 8$) it reduced the tension response without significantly affecting the urothelium/lamina propria frequency response (Figure 4-2).

4.4.3 *The effect of clinical anticholinergics on the muscarinic response to carbachol*

At a concentration of 100 nM darifenacin, oxybutynin, and tolterodine significantly reduced the frequency and tension responses of urothelium/lamina propria strips to carbachol (1 μM). Solifenacin (100 nM) slightly inhibited the response to carbachol, although this was not statistically significant at this concentration. At a concentration of 1 μM all four antagonists significantly depressed the urothelium/lamina propria frequency and tension responses to carbachol (Figure 4-3).

4.4.4 *Urothelium/lamina propria frequency responses to stretch*

In separate experiments, stretching the urothelium/lamina propria strips elicited an increase in the rate of spontaneous phasic contractions of $19 \pm 3\%$ ($P < 0.001$) and baseline tension also increased from $1.75 \pm 0.12\text{g}$ to $9.58 \pm 0.80\text{g}$ ($P < 0.001$, $n = 42$). The increase in frequency of phasic contractions induced by stretch was not significantly altered after desensitisation of P_{2X} receptors with $\alpha\beta\text{mATP}$ ($10\text{ }\mu\text{M}$, $n = 12$), the presence of the NOS inhibitor L-NNA ($100\text{ }\mu\text{M}$, $n = 7$) or the cyclooxygenase inhibitor indomethacin ($5\text{ }\mu\text{M}$, $n = 12$). However the stretch-induced increase in frequency was abolished by atropine ($1\text{ }\mu\text{M}$, $n = 11$, $P < 0.01$, Figure 4-4), indicating the involvement of muscarinic receptors. To identify which receptor mediated this response, the frequency response to stretch was examined in the presence of selective antagonists (Figure 4-4). In the presence of 4-DAMP (10 nM , $n = 7$) the increase in frequency observed during stretch was abolished. In contrast, pirenzepine (100 nM , $n = 7$) and methoctramine (100 nM , $n = 8$) had no effect on the stretch-induced increase in phasic contractions.

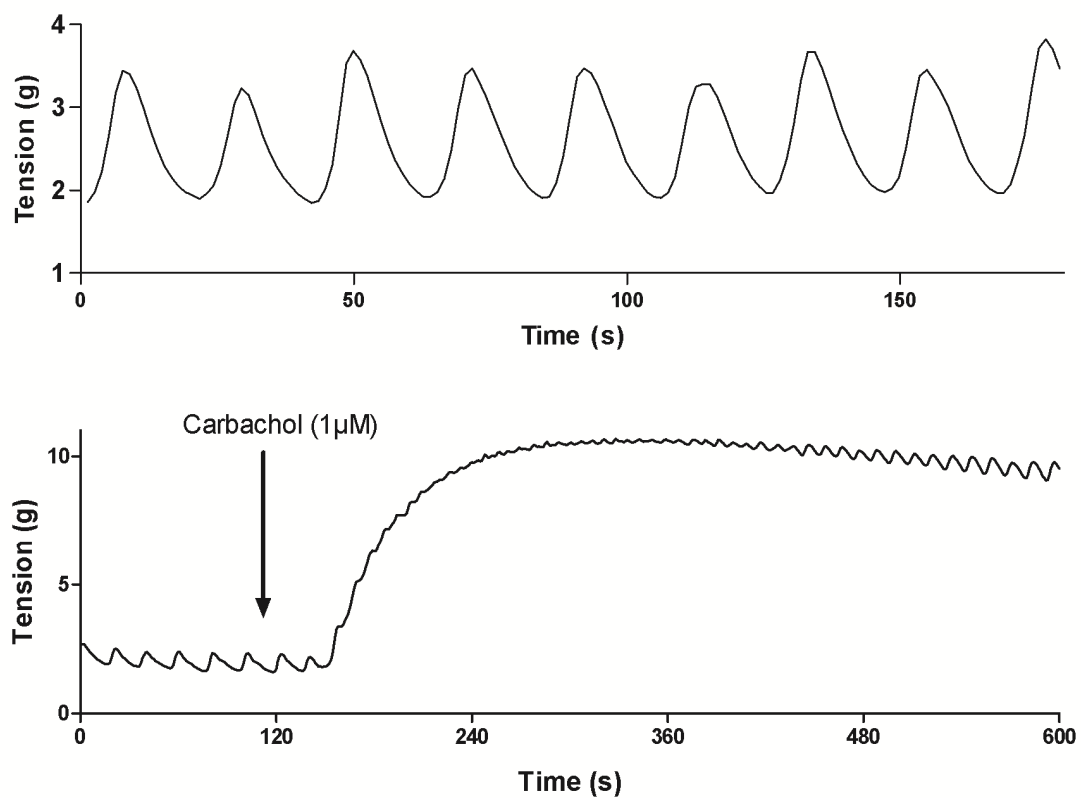


Figure 4-1: Spontaneous activity of isolated urothelium/lamina propria strips; upper trace in the absence of any drug and lower trace in the presence of carbachol (1 μ M). Carbachol induced increases in baseline tension, but also increased the frequency of spontaneous contractions.

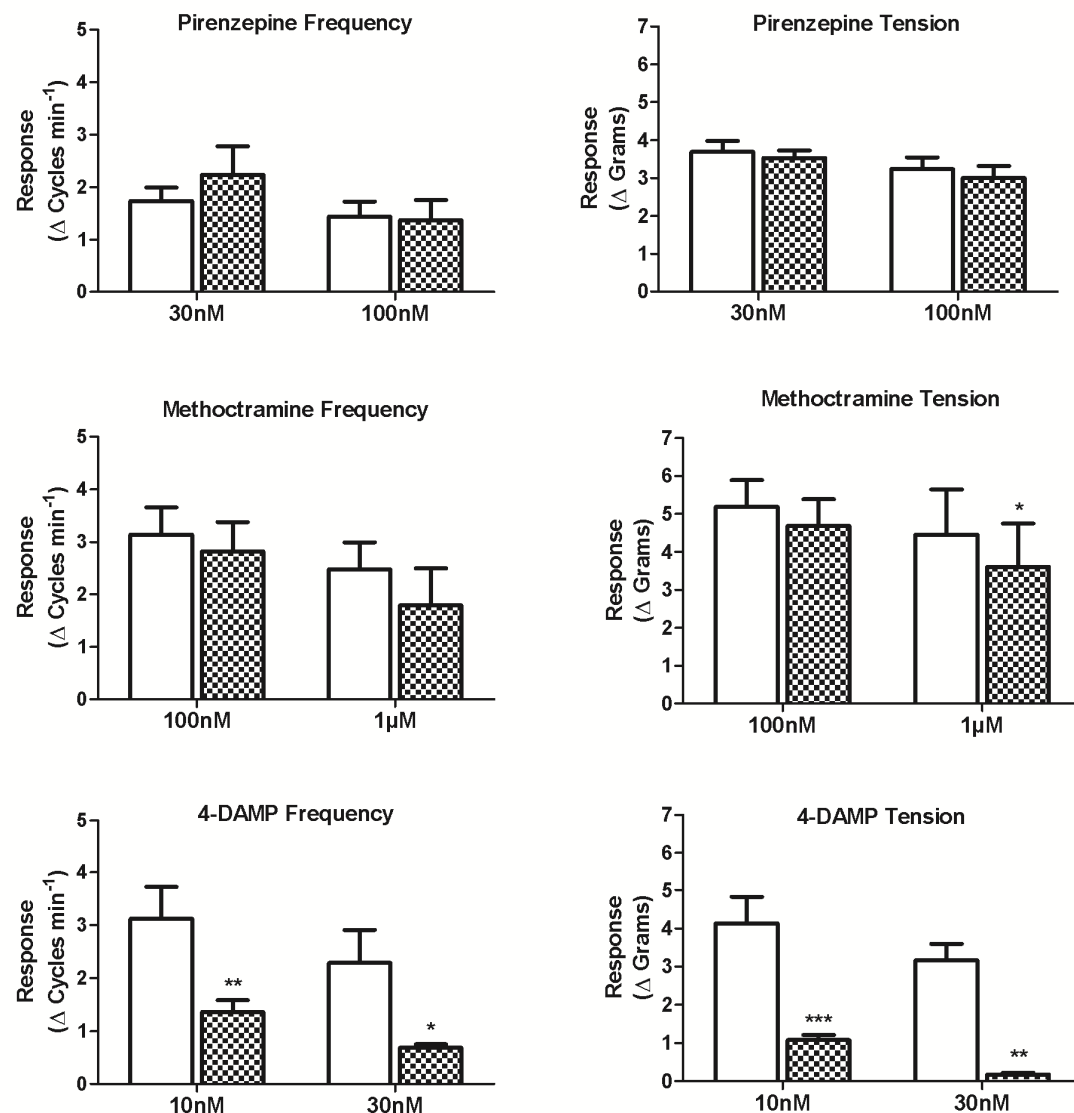


Figure 4-2: Increases in urothelium/lamina propria contractile frequency induced by carbachol (1 μ M). Responses were obtained to carbachol in the absence (open columns) and in the presence of antagonists (shaded columns). Upper panels show changes in frequency (cycles min⁻¹) and baseline tension (grams) to carbachol in the absence and presence of 30 nM and 100 nM pirenzepine (M₁ selective antagonist); middle panels show the effects of 100 nM and 1 μ M methoctramine (M₂ selective antagonist) and lower panels show the effects of 10 nM and 30 nM 4-DAMP (M₃ selective antagonist). *P < 0.05, **P < 0.01, ***P < 0.001.

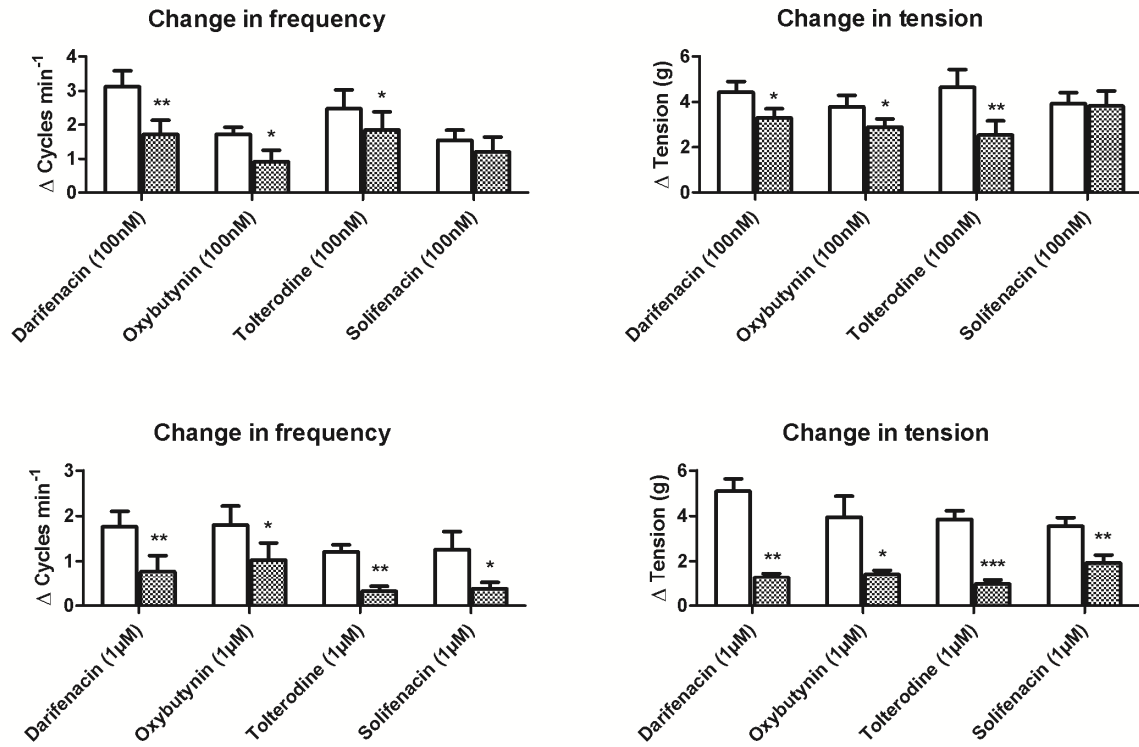


Figure 4-3: Effects of clinically used anti-muscarinic drugs on frequency (left panels) and tension responses (right panels) of isolated urothelium/lamina propria strips to carbachol (1 μ M). Increases in urothelial frequency (cycles min⁻¹) and tension (grams) induced by carbachol are shown in the absence (open columns) and in the presence of antagonists (shaded columns) at concentrations of 100 nM (upper panels) and 1 μ M (lower panels). *P < 0.05, **P < 0.01, ***P < 0.001.

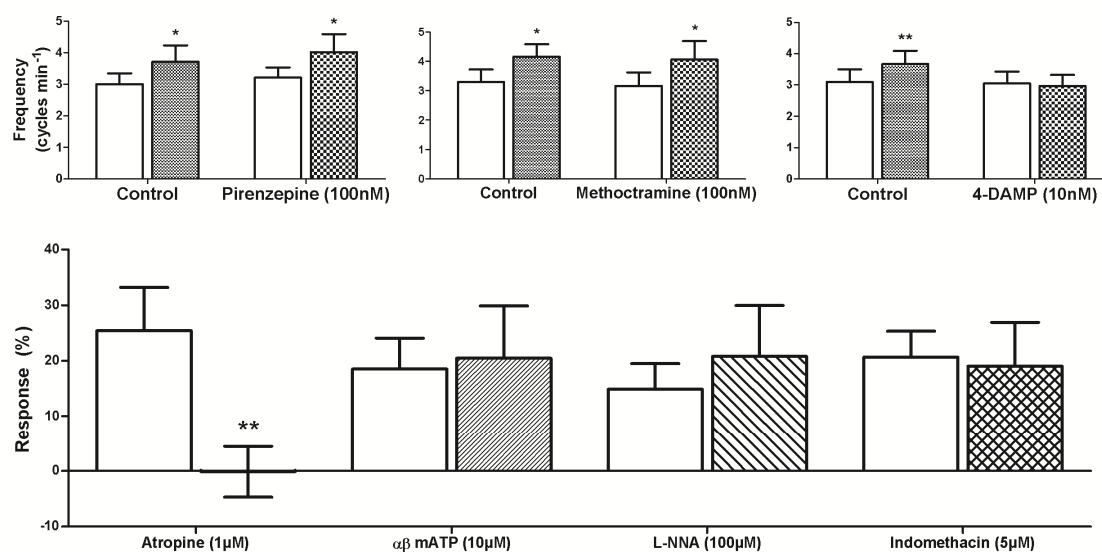


Figure 4-4: Upper panels: Effects of muscarinic receptor subtype selective antagonists on the frequency responses of urothelium/lamina propria strips to stretch. Lower panels: Increases in the frequency of spontaneous contractions of isolated strips of urothelium/lamina propria when stretched in the absence (open columns) and presence (shaded columns) of atropine (1 μM), α,β-methylene ATP (10 μM), L-NNA (100 μM) or indomethacin (5 μM). Responses are expressed as the percentage increase in frequency induced by stretch compared to those in the absence of any drug. The spontaneous frequency before (open columns) and after stretching (solid columns) was obtained in the absence and then presence of either pirenzepine (M₁-selective), methoctramine (M₂-selective) or 4-DAMP (M₃-selective). Stretching significantly increased the frequency of spontaneous urothelium/lamina propria contractions in all groups, except in the presence of 4-DAMP *P < 0.05, **P < 0.01.

4.5 DISCUSSION

Contraction of the lamina propria was first reported for tissues from the urethra of the rabbit (Mattiasson et al., 1985a, Zygmunt et al., 1993) where a number of agonists and electrical field stimulation were shown to induce contractions. In a recent study our laboratory showed that isolated strips of urothelium/lamina propria from the pig bladder dome can also contract in response to neurokinin-A and muscarinic receptor stimulation, the responses to neurokinin-A being mediated via NK2 receptors (Sadananda et al., 2008). These tissues from the dome of the bladder rarely develop spontaneous phasic contractions. This spontaneous activity was observed in all urothelium/lamina propria preparations but was generally absent in detrusor muscle strips, with only 25% of detrusor strips demonstrating any spontaneous contractile activity. Furthermore, in detrusor strips that did display this phenomenon, the rate of spontaneous contractions was significantly slower than that of the urothelium/lamina propria. The activity was insensitive to tetrodotoxin, which makes it unlikely to be neuronally mediated. Whether the spontaneous activity originates in the smooth muscle or from another cell type is not clear. A network of interstitial cells exists in the lamina propria and it has been suggested that these cells are similar to the Interstitial Cells of Cajal which act as pacemakers of electrical and contractile activity in the gastrointestinal system (Fry et al., 2007, Ikeda et al., 2009, Ward and Sanders, 2001). Electrophysiological studies have shown that the interstitial cells of the bladder lamina propria may develop spontaneous intracellular calcium and membrane potential transients and can function as a syncytium (Fry et al., 2007). The present results suggest that this cellular activity may be involved, and is potentially translated into contractile activity at an intact tissue level resulting in spontaneous phasic contractions, the frequency of which was increased by carbachol. Of interest is the report that the frequency of transient calcium and membrane potential changes in bladder interstitial

cells is also increased during muscarinic receptor stimulation (Fry et al., 2007). In some tissues activation of muscarinic receptors stimulates the production of prostaglandins (Hara et al., 2009) or nitric oxide (Andersson et al., 2008), however the urothelium/lamina propria contractile responses to carbachol were insensitive to indomethacin and L-NNA.

The detrusor smooth muscle possesses a mixed population of M₂ and M₃ muscarinic receptors, with a M₂:M₃ ratio of about 3:1 in most species (Yamanishi et al., 2002a, Chess-Williams, 2002a). However the responses of isolated detrusor strips to muscarinic agonists are mediated via the minor population of M₃ receptors (Sellers et al., 2000b, Chess-Williams et al., 2001, Fetscher et al., 2002). A similar result was obtained in the urothelium and lamina propria. Molecular biology and radioligand binding studies indicate that M₂ receptors predominate at the mRNA and protein level in this tissue (Mansfield et al., 2005, Bschleipfer et al., 2007), but frequency responses to carbachol were significantly reduced by the M₃ selective antagonist 4-DAMP. The M₁ selective antagonist pirenzepine had no effect on responses, whilst the M₂ selective antagonist methoctramine, had no effect at lower concentrations and even at 1 µM only had a minor effect on tension but not frequency responses. This effect of methoctramine on tension responses to carbachol would be due to a lack of selectivity for this antagonist at the higher concentration resulting in M₃ receptor antagonism with the 1 µM concentration. In contrast, 4-DAMP significantly reduced frequency and tension responses to carbachol at a very low concentration (10 nM), indicating the increase in the rate of phasic spontaneous contractions of the urothelium/lamina propria to carbachol was mediated via the M₃ receptor subtype.

The relevance of the spontaneous contractile activity is unknown. Detrusor overactivity results from involuntary contractions demonstrated by cystometry during the filling stage

where the bladder, including the urothelium, undergoes periods of stretch. Thus it is possible that the contractile pacemaker activity in the urothelium/lamina propria may be activated during stretch and may possibly then drive contractions of the detrusor. To investigate this possibility, we examined the effects of stretching the tissues and found that the frequency of the phasic contractile activity was increased. This effect could be direct on the cells initiating the phasic contractions; whether smooth muscle, interstitial cells, or another cell type sensitive to stretch; or the activity might be driven by factors being released from the urothelium/lamina propria during stretch.

To examine the latter hypothesis, a number of factors known to be released from the urothelium during stretch were investigated including ATP, acetylcholine (ACh) and nitric oxide (Birder et al., 2010b, Smith et al., 2008). Although NO is usually inhibitory, there is evidence to suggest that it can exert excitatory effects in the bladder (Gillespie and Drake, 2004). Stretching tissues resulted in an increase in the frequency of phasic contractions, but this was not altered in the presence of L-NNA. Furthermore, desensitising P2X purinergic receptors with α,β -methyleneATP had no effect on the stretch-induced responses, whereas atropine completely abolished the frequency response to stretch indicating that ACh released from the urothelium/lamina propria was responsible for the stretch-induced increases in spontaneous rate. In support of this, 4-DAMP depressed these responses, whilst methoctramine and pirenzepine failed to affect frequency responses to stretch, again indicating that responses were mediated via the M_3 receptor subtype. Thus, stretch appears to induce the release of ACh from the urothelium/lamina propria which then activates M_3 receptors on the cells initiating the spontaneous contractile activity.

There is some evidence to suggest that this activity in the urothelium/lamina propria may be able to drive detrusor smooth muscle activity. Firstly, electrophysiological studies in the rat have shown that the calcium and membrane potential transients produced by carbachol begin near the urothelial-lamina propria interface before spreading to the detrusor (Zygmunt et al., 1993). Secondly, when bladder sheets were examined, spontaneous contractions were greater in bladder tissue with an intact urothelium/lamina propria (Sui et al., 2008). Finally, the increased contractions recorded in the bladders of cats with feline cystitis have been shown to be associated with enhanced calcium transients and supersensitivity to muscarinic stimulation in the urothelium/lamina propria (Ikeda et al., 2009). Thus, it appears possible that spontaneously active cells in the lamina propria may be able to drive detrusor contractions, at least in the overactive bladder where gap junctions are increased (Roosen et al., 2009). Future studies will be required to test this hypothesis.

Currently, the main treatments for overactive bladder are muscarinic antagonists. The exact clinical mechanisms of action for these drugs is unclear, however this study has shown that these drugs will antagonise the muscarinic receptors on the cells regulating urothelium/lamina propria contractile activity, thus preventing these actions of acetylcholine released during stretch. Tolterodine, solifenacin, darifenacin and oxybutynin all depressed the increases in urothelium/lamina propria frequency and tension induced by carbachol. Thus, the responses of the urothelium/lamina propria may represent another possible site of action for these clinically effective anti-muscarinic agents.

4.6 CONCLUSIONS

The urothelium/lamina propria exhibits spontaneous phasic contractile activity which is increased during stretch. The mechanism appears to involve acetylcholine and M₃ muscarinic receptors. Anticholinergic drugs used clinically depressed these responses and this mechanism may represent an additional site of action for these drugs in the treatment of bladder overactivity.

Chapter 5

Contractile activity of the bladder urothelium/lamina propria and its regulation by nitric oxide

The manuscript in the following chapter is presented as a pre-publication copy, prior to the reviewer and publisher's requested alterations. The final version of this manuscript can be accessed online from the journal. Formatting and terminology changes have been made to maintain consistency throughout this thesis.

All data presented in this manuscript is the sole work of C. Moro

Published manuscript

Moro C, Leeds C, Chess-Williams R (2012). Contractile activity of the bladder urothelium/lamina propria and its regulation by nitric oxide. *Eur J Pharmacol.* 15;674(2-3):445-9

Published abstracts and conference presentations arising from this chapter:

Moro C, Chess-Williams R (2009). The affect of nitric oxide on urothelial contractile activity. *Proceedings of the 43rd Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists Scientific Meeting* 43.

Moro C, Chess Williams R (2009). Effects of autonomic agonists and nitric oxide donors on urothelial spontaneous contractile activity. *Proceedings of the 1st National Symposium on Advances in Urogenital and Gut Research* 1(1): 16.

5.1 ABSTRACT

In the bladder, nitric oxide (NO) is released from neuronal and non-neuronal sources, but its actions are unclear. Strips of urothelium plus lamina propria contract in response to agonists and develop spontaneous phasic contractions, and the aim of this study was to investigate the influence of NO on this activity. Isolated strips of urothelium/lamina propria from porcine bladder developed spontaneous contractions (3.54 ± 0.30 cycles min^{-1}) and contracted in response to carbachol and electrical field stimulation (EFS). The NO synthase inhibitor N $^{\omega}$ -nitro-L-arginine (L-NNA, 100 μM) had no effects on the tissues, but the NO donors diethylamine NONOate (DEANO, 100 μM) and nitroprusside (10 μM) caused relaxation, slowed the spontaneous rate of contractions and inhibited responses to carbachol. Maximum tonic contractions to carbachol were reduced by $17 \pm 4\%$ ($P < 0.001$) and $35 \pm 5\%$ ($P < 0.001$) by DEANO and nitroprusside respectively and the potency of carbachol was also reduced. Carbachol also increased the spontaneous frequency of contraction and these rate responses were again inhibited by DEANO and nitroprusside, but unaffected by L-NNA. Similarly, responses to EFS were significantly depressed (52 - 70%) by DEANO ($P < 0.05$), but were unaffected by L-NNA. These data demonstrate spontaneous contractile activity and also nerve and agonist-induced tonic contractile activity within the urothelium and lamina propria. This activity is sensitive to depression by NO, but NO does not appear to be spontaneously released to influence this activity, nor does it appear to be released by muscarinic receptor stimulation. However, the results suggest that in situations where NO production is increased, NO can influence the contractile activity of this tissue.

5.2 INTRODUCTION

Nitric oxide (NO) plays a major role in the bladder outlet region during micturition, inducing relaxation and preventing large increases in intravesical pressure during bladder contraction (Bustamante et al., 2010). Nitric oxide is also produced in the bladder body itself, although its role in this region of the bladder is less clear. Neuronal NO synthase (nNOS) has been identified in the nitrergic nerves of the lamina propria in the human bladder and also between smooth muscle cells (Fathian-Sabet et al., 2001). Along with acetylcholine, NO is released as a co-transmitter from parasympathetic nerves and exerts an inhibitory effect on contraction (Dokita et al., 1991). Thus in electrical field stimulated (EFS) tissues, inhibition of NO synthase enhances contraction amplitude (Garcia-Pascual et al., 1991), while in precontracted tissues, NO donors induce relaxation (Hernandez et al., 2008). However the influence of NO on detrusor contraction is relatively minor compared with its actions on the bladder outlet region (Kedia et al., 2009).

Recently the urothelium and underlying lamina propria (urothelium/lamina propria) have been shown to be alternative sources of NO in the bladder. Endothelial NO synthase (eNOS) has been identified using immunohistochemistry in the urothelium of the rat (Giglio et al., 2005) and adrenergic stimulation has been shown to release NO from urothelial cells in culture (Birder et al., 1998). Furthermore, extra-neuronal nNOS has been found in the urothelium and the interstitial cells of the lamina propria (De Ridder et al., 1999). The function of this non-neuronal NO is uncertain, but it may play a role in modulating sensory nerve activity (Aizawa et al., 2011). Other targets of NO action in the bladder have been identified using cGMP immunoreactivity and include detrusor, epithelial and interstitial cells (Fathian-Sabet et al., 2001, Gillespie et al., 2006a). Although the vast majority of reports

concerning NO have indicated that it has an inhibitory effect on bladder smooth muscle, there is also some evidence to suggest that NO can exert excitatory effects. In the isolated mouse bladder it has been shown that NO can increase phasic contractile activity (Gillespie and Drake, 2004) and in pre-contracted human detrusor strips both relaxation and contraction responses to NO donors have been observed (Moon, 2002). Thus, NO is produced within the bladder wall, but its actions and role in bladder function remain unclear.

In addition to contraction of the detrusor smooth muscle, isolated strips of urothelium (inner epithelial layer of the bladder) and underlying lamina propria from the dome of the pig bladder (Sadananda et al., 2008) and also strips of lamina propria from the rabbit urethra have previously been shown to contract to agonists (Mattiasson et al., 1985a). These tissues, urothelium/lamina propria, from the dome of the pig bladder also spontaneously develop phasic contractile activity (Moro and Chess-Williams, 2010). The aims of the present study were to investigate whether NO has inhibitory or excitatory effects on this tissue. The effects of NO donors and a NO synthase inhibitor were examined on frequency and tension responses of strips of porcine urothelium/lamina propria.

5.3 MATERIALS AND METHODS

5.3.1 Tissue preparation

Fresh pig bladders from pigs (5 - 6 months age, ~50 kg) were obtained from a local abattoir and immediately immersed in Krebs-bicarbonate solution composed of NaCl (188.4 mM), NaHCO₃ (24.9 mM), CaCl₂ (1.9 mM), MgSO₄ (1.2 mM), KH₂PO₄ (1.2 mM) and glucose (11.7 mM). The bladder was opened longitudinally and strips of urothelium plus lamina

propria (20 x 5 mm) were dissected from the anterior wall of the dome of the bladder. The urothelium/lamina propria is easily recognised in the pig bladder, being pale in colour compared to the underlying detrusor smooth muscle. The strips of tissue were set up under 2g tension in baths containing Krebs-bicarbonate solution at 37°C and gassed with 5% CO₂ in oxygen. Isometric tension developed by tissues was recorded to PC via a Powerlab recording system and Labchart software (AdInstruments, Castle Hill, Australia).

5.3.2 Contractile responses to electrical field stimulation

Preliminary experiments indicated that the optimal stimulation parameters for tissue strips was 5 second trains of pulses (0.5 ms duration, 30v) applied every 100 seconds. Two stimulation frequencies (10 and 20 Hz) were examined and the neurotoxin tetrodotoxin (1 µM) was used to confirm the neurogenic origin of contractions. Responses to electrical stimulation at both frequencies were recorded before and after the addition of either N_ω-nitro-L-arginine (L-NNA, 100 µM), diethylamine NONOate (DEANO, 100 µM) or sodium nitroprusside (10 µM).

5.3.3 Frequency responses to carbachol

To investigate the influence of NO on muscarinic receptor mediated rate responses, separate groups of tissues were stimulated with a single concentration of carbachol (1 µM) and responses re-examined in the presence of either L-NNA (100 µM), DEANO (100 µM) or sodium nitroprusside (10 µM).

5.3.4 *Contractile responses to carbachol*

Complete cumulative concentration-response curves to carbachol were obtained for tonic contractile responses. After washout of the drug, concentration-response curves to carbachol were repeated in the presence of either L-NNA (100 μ M), DEANO (100 μ M) or sodium nitroprusside (10 μ M). Control experiments were performed without the addition of NO synthase inhibitor or NO donor and these experiments were used to correct for any time-dependent changes in tissue sensitivity or responsiveness during the course of the experiment.

5.3.5 *Drugs*

Carbamoylcholine chloride (carbachol), diethylamine NONOate (DEANO), N_ω-nitro-L-arginine (L-NNA), sodium nitroprusside and tetrodotoxin were purchased from Sigma-Aldrich Co. (St. Louis, MO).

5.3.6 *Data analysis*

Graphpad PRISM software was used for statistical analyses. Mean (\pm S.E.M.) increases in tension in grams and increases in spontaneous phasic contractions in cycles min⁻¹ were calculated. Where complete concentration-response curves were obtained, mean (\pm S.E.M.) maximum responses and -logEC₅₀ (pEC₅₀) values were calculated. Student's paired *t*-tests were used to compare responses obtained in the absence and presence of NO synthase inhibitor or NO donors.

5.4 RESULTS

5.4.1 *Spontaneous tissue activity*

Within 10 min of being set up in the baths, the isolated strips of urothelium/lamina propria spontaneously developed phasic contractile activity. The contractions occurred with a frequency of 3.54 ± 0.26 cycles min^{-1} , a baseline tension of $1.99 \pm 0.09\text{g}$ and an amplitude of $0.73 \pm 0.07\text{g}$ ($n = 59$). The addition of the NO synthase inhibitor L-NNA ($100 \mu\text{M}$) alone had no effect on these resting values. In contrast, both the NO donors, DEANO (Figure 5-1) and nitroprusside, exerted significant inhibitory effects on the tissues. When applied to unstimulated tissues, DEANO ($100 \mu\text{M}$, $n = 17$) caused relaxation of the urothelial strips by $40 \pm 6\%$ ($P < 0.001$) and slowed spontaneous phasic contractions by $58 \pm 11\%$ ($P < 0.001$). The amplitude of phasic contractions was also reduced by $39 \pm 10\%$ ($P < 0.01$). Nitroprusside ($10 \mu\text{M}$, $n = 9$) had a similar effect, relaxing the tissues by $32 \pm 5\%$ ($P < 0.001$) and slowing the rate of spontaneous contractions by $35 \pm 14\%$ ($P < 0.05$), but without significantly reducing the amplitude of spontaneous contraction (Figure 5-2).

5.4.2 *Responses to carbachol*

Carbachol ($1 \mu\text{M}$) induced tonic contractions with the basal tension increasing by $4.49 \pm 0.86\text{g}$ ($n = 23$). The frequency of spontaneous phasic contractions was also increased by 3.41 ± 0.29 cycles min^{-1} ($P < 0.001$). The addition of L-NNA alone had no effect on resting values and the NO synthase inhibitor also failed to influence subsequent responses to carbachol. Increases in basal tension and spontaneous rate induced by carbachol ($1 \mu\text{M}$) in the presence of L-NNA ($100 \mu\text{M}$, $n = 7$) were similar to paired controls (Figure 5-3). The

addition of DEANO significantly inhibited responses to carbachol. In the presence of DEANO (100 μ M) both the frequency responses and the baseline tension responses to carbachol (1 μ M) were significantly reduced (both $P < 0.001$, $n = 20$). Similarly, nitroprusside inhibited the increases in frequency and tension induced by carbachol (Figure 5-3).

5.4.3 *Carbachol concentration-contraction curves*

For contractile responses it was possible to obtain complete concentration-response curves to carbachol and the effects of L-NNA (100 μ M), DEANO (100 μ M) and nitroprusside (10 μ M) could be investigated (Figure 5-4). As with the experiments using a single concentration of carbachol, L-NNA (100 μ M) was without effect and concentration-response curves in the absence and presence of L-NNA were identical. Again, both DEANO and nitroprusside exerted significant inhibitory effects. The potency (pEC_{50} value) of carbachol was reduced from 6.18 ± 0.08 to 4.95 ± 0.06 in the presence of DEANO (100 μ M, $n = 8$, $P < 0.001$) and from 5.91 ± 0.04 to 5.11 ± 0.03 by nitroprusside (10 μ M, $n = 8$, $P < 0.001$). Both drugs significantly reduced the maximum contractile responses to carbachol, DEANO by $17 \pm 4\%$ ($n = 8$, $P < 0.001$), and nitroprusside by $35 \pm 5\%$ ($n = 8$, $P < 0.001$)

5.4.4 *Responses to electrical field stimulation*

When stimulated electrically at 10 Hz, urothelium/lamina propria strips contracted by 0.79 ± 1.51 g ($n = 6$) above the baseline spontaneous contractions. Increasing the frequency of stimulation to 20 Hz further increased the contractions to 1.12 ± 0.16 g ($P < 0.001$). In the presence of tetrodotoxin (1 μ M), responses were reduced by $79 \pm 12\%$ at 10 Hz ($n = 6$, $P <$

0.001) and by $80 \pm 26\%$ at 20 Hz ($P < 0.01$), indicating that they were significantly neurogenic in nature.

The addition of L-NNA (100 μ M, $n = 8$) had no effect on the responses of urothelium/lamina propria tissues to electrical field stimulation (Figure 5-3). In contrast, when detrusor smooth muscle strips were stimulated using identical stimulation parameters, L-NNA caused an increase in contraction magnitude by $65 \pm 27\%$ at 10 Hz and $56 \pm 27\%$ at 20 Hz ($n = 8$, both $P < 0.05$). Also, the time taken to relax back to the baseline tension was increased by L-NNA in the detrusor smooth muscle, but not the urothelium/lamina propria. Relaxation time for detrusor muscle was increased by $34 \pm 9\%$ at 10 Hz ($P < 0.01$) and $10 \pm 4\%$ at 20 Hz ($n = 8$, $P < 0.05$).

In urothelium/lamina propria tissues, baseline tension was reduced on the addition of the NO donors; DEANO reducing tension by $64 \pm 4\%$ ($n = 6$, $P < 0.001$) and nitroprusside by $24 \pm 2\%$ ($n = 6$, $P < 0.001$). DEANO (100 μ M, $n = 6$), but not nitroprusside (10 μ M, $n = 6$), also significantly inhibited contractile responses of urothelium/lamina propria strips to electrical field stimulation (Figure 5-5). DEANO (100 μ M) reduced responses by $70 \pm 33\%$ when tissues were electrically stimulated at 10 Hz ($P < 0.05$) and by $52 \pm 9\%$ when tissues were stimulated at 20 Hz ($P < 0.01$).

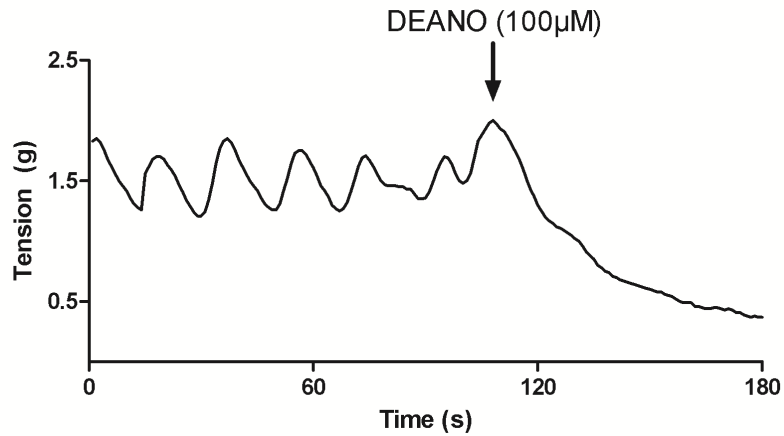


Figure 5-1: Experimental trace showing the effects of DEANO (100 μM) on the contractile activity of unstimulated strips of urothelium and lamina propria.

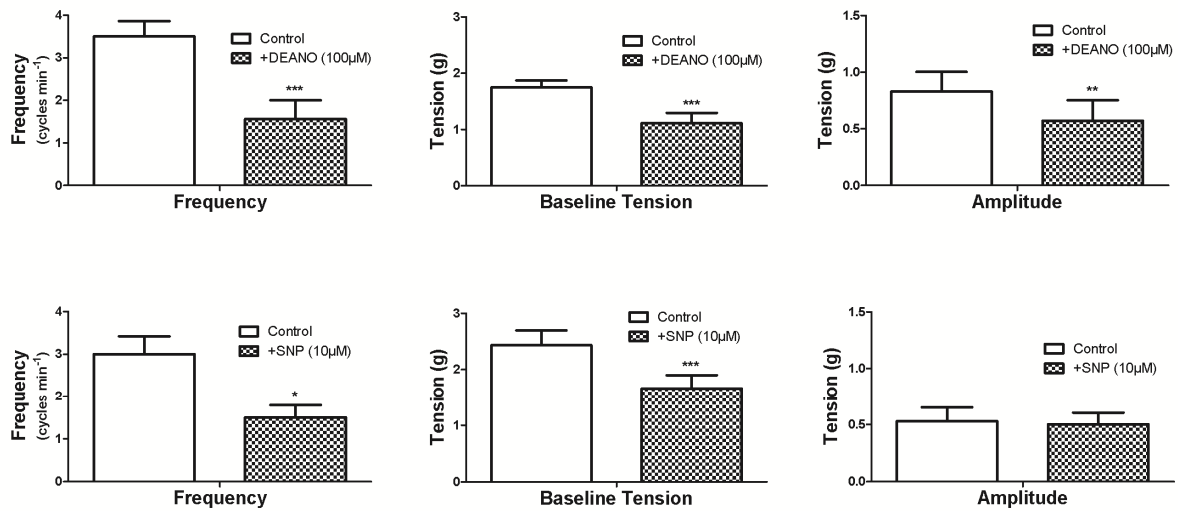


Figure 5-2: Effects of DEANO (top row, 100 μM , $n = 17$) and sodium nitroprusside (SNP, bottom row, 10 μM , $n = 9$) on the spontaneous frequency (cycles min^{-1}), baseline tension (g) and amplitude of phasic contractions (g) of strips of urothelium/lamina propria. * $P < 0.01$, ** $P < 0.01$, *** $P < 0.001$.

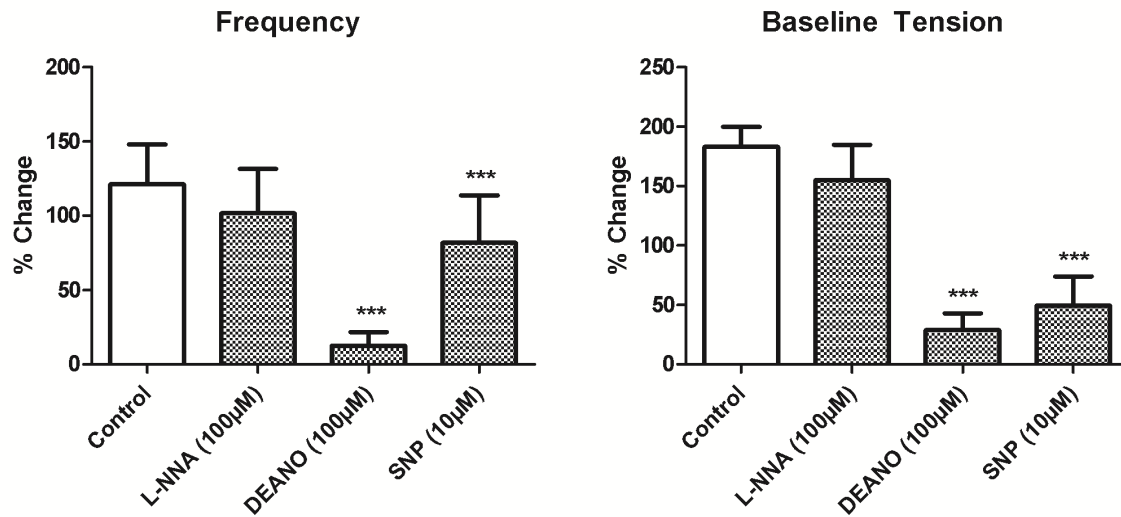


Figure 5-3: Frequency and baseline tension responses of strips of urothelium/lamina propria to carbachol (1 μ M, n = 23) in the absence (open column) and in the presence of L-NNA (100 μ M, n = 7), DEANO (100 μ M, n = 20) and sodium nitroprusside (SNP, 10 μ M, n = 12). ***P < 0.001 compared with control responses.

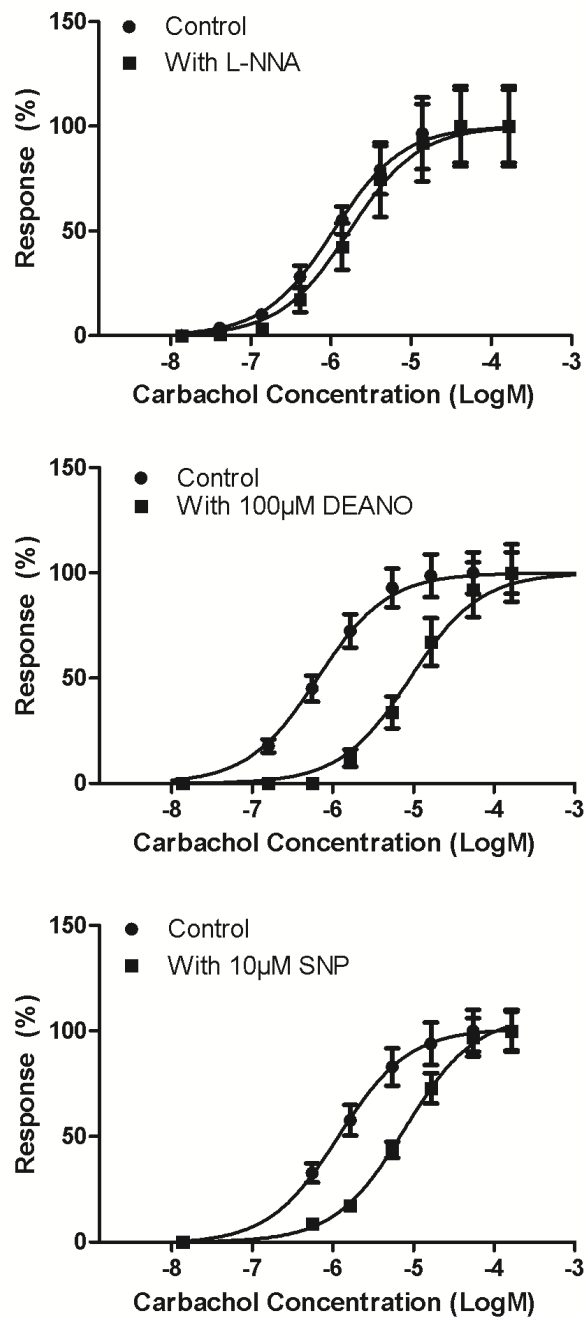


Figure 5-4: Cumulative concentration-response curves to carbachol of urothelial/lamina propria strips in the absence and presence of L-NNA (100 μ M, upper panel), DEANO (100 μ M, centre panel) and sodium nitroprusside (SNP, 10 μ M, lower panel). Responses are expressed as a percentage of the maximum response in individual experiments. (n = 8 for all groups).

		Stimulation at 10 Hz		Stimulation at 20 Hz	
Tissue	n	Control	L-NNA	Control	L-NNA
Urothelium	12	1.92±0.27g	2.00±0.25g	2.37±0.41g	2.33±0.33g
Detrusor	8	4.17±1.12g	6.20±1.37g ^a	10.99±2.97g	14.83±3.14g ^a

Table 9: Contractile responses of urothelium/lamina propria and detrusor strips to electrical field stimulation at 10 Hz and 20 Hz in the absence and presence of L-NNA (100 µM). ^aP < 0.05

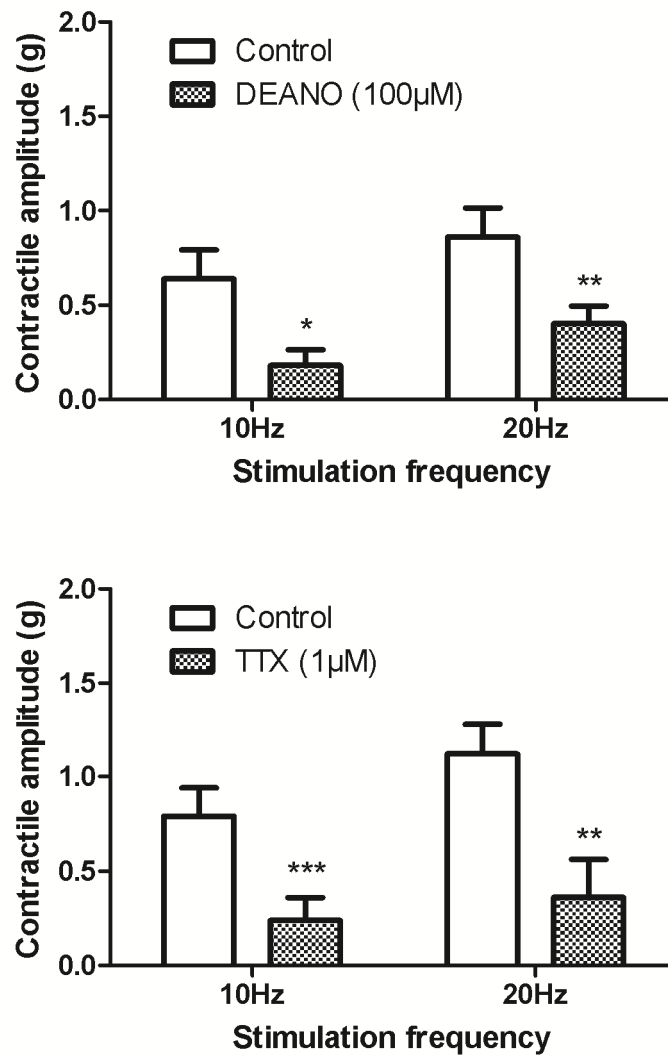


Figure 5-5: Contractile responses to electrical field stimulation (10 Hz and 20 Hz) in the absence and presence of DEANO (100 μM, n = 6) and tetrodotoxin (1 μM, n = 6). *P < 0.01, **P < 0.01, ***P < 0.001

5.5 DISCUSSION

The tissue strips of urothelium and lamina propria developed spontaneous contractile activity. Whether these contractions are developed by smooth muscle within the lamina propria or whether they are elicited by another cell type is uncertain. It has previously been suggested that the tonic contractions of these tissues induced by NKA involve interstitial cells, since tissues were able to contract in response to NKA even after removal of smooth muscle by microscopic dissection (Sadananda et al., 2008). In the present study, full thickness strips of urothelium and lamina propria were investigated and contractions may represent the activity of some remaining detrusor muscle, interstitial cells or a muscularis mucosa in the lamina propria. Of particular interest however, was the finding that these tissues developed spontaneous contractions and the frequency of contractions was increased by carbachol. The interstitial cells of the bladder resemble the Interstitial Cells of Cajal of the gastrointestinal system, where they act as pacemakers of electrical and contractile activity. Interstitial cells have been identified in the bladder lamina propria of several species where they spontaneously generate transient membrane potentials and calcium transients (Kanai et al., 2007). The results of the present study suggest that these cellular transients are transduced into whole tissue contractile activity resulting in spontaneous phasic activity. Interstitial cells are also found distributed amongst the detrusor muscle fibres, but in the urothelium they are arranged in an organised network. It is interesting that the spontaneous contractile activity was observed in all the urothelial strips, but it only occurred in a minority (25%) of detrusor muscle strips. This urothelial phasic activity persisted after the addition of tetrodotoxin (1 μ M) and atropine (1 μ M) indicating that neuronal inputs were not necessary for its generation.

Both NO donors caused a reduction in basal tone and simultaneously slowed the rate of spontaneous contractions. This indicates that the cells responsible for eliciting contraction and the pacemaker cells initiating the spontaneous activity, have the intracellular machinery to respond to NO. Gillespie *et al* (2006) have identified cells in the lamina propria that stain for cGMP following the addition of NO donors and these cells also stained for markers of interstitial cells. These cells therefore have the intracellular mechanisms to respond to NO and the current study demonstrates that NO can inhibit contraction and pacemaker activity in this tissue. Interestingly the NO synthase inhibitor L-NNA did not affect contractile activity suggesting that there was no basal release of NO in these tissues. This would appear to apply to both the contractile cells and those initiating the spontaneous activity.

Since several studies have shown that NO release in the bladder can be due to muscarinic receptor stimulation (Munoz *et al.*, 2010, Kullmann *et al.*, 2008), the role of NO in modulating tissue responses to carbachol was also examined. Carbachol increased the rate of phasic contractions, which agrees with reports of interstitial cells also responding to carbachol with increases in the frequency of intracellular calcium transients (Kanai *et al.*, 2007). Radioligand binding studies have shown that the urothelium/lamina propria of the pig possesses a greater density of muscarinic receptors than the detrusor muscle (Hawthorn *et al.*, 2000), and the predominant receptor subtype is the M₂ receptor (Yamanishi *et al.*, 2000). Despite this finding, the tonic contractions of this tissue appear to be mediated via M₃ receptors and this receptor also mediates the carbachol induced increases in the rate of spontaneous phasic contractions (Moro and Chess-Williams, 2010)). This is not unusual, since responses of the detrusor smooth muscle have also been shown to be mediated via the minor population of M₃ receptors (Sellers *et al.*, 2000b).

In urothelium/lamina propria tissues the inhibition of NO synthase did not affect contractile or frequency responses to carbachol. However the NO donors inhibited both these responses to carbachol and also inhibited the contractile responses to electrical field stimulation. The results again suggest that these tissues respond to NO, but there was no basal release or muscarinic receptor-stimulated release of NO. This contrasts with the detrusor strips where L-NNA potentiated responses to electrical field stimulation, suggesting that NO was released and acted in the detrusor during nerve stimulation. It has been suggested that in the rat, urothelial muscarinic receptors induce NO release but this only occurs after the induction of inducible NO synthase in conditions such as bladder inflammation (Giglio et al., 2005). Situations where the expression of this enzyme in the urothelium is increased include interstitial cystitis (Koskela et al., 2008) and bladder outlet obstruction (Romih et al., 2003). In these conditions NO may be released and would exert the NO effects observed in the present study.

It seems likely that NO is synthesized predominantly in the lamina propria since there is only sparse NOS distribution in the urothelium (Fathian-Sabet et al., 2001, Persson et al., 1999). Munoz et al. (2010) also reported that NO release induced by electrical field stimulation in the rat, was not affected by removal of the urothelium. The targets of NO action have also been identified in immunohistochemical studies. In the presence of NO donors, cGMP staining cells have been demonstrated in the detrusor and nerves of the bladder but also in the urothelium and the interstitial cells of the lamina propria (Fathian-Sabet et al., 2001, Gillespie et al., 2006a). These cells may possibly be the target of NO in the present experiments.

5.6 CONCLUSIONS

The urothelium and lamina propria develops spontaneous contractile activity that is inhibited by NO donors. The tissues contract in response to electrical field stimulation and respond to carbachol with an increase in tonic baseline tension and an increase in spontaneous contractile rate, and all of these responses are sensitive to inhibition by NO donors. However, inhibition of NO synthase had no effect on responses suggesting that there is no basal release of NO nor any muscarinic receptor mediated release of NO in these tissues. However the potency of NO in these tissues suggests that in conditions where NO release is enhanced it will have significant effects on urothelial function.

Chapter 6

Adrenoceptor function and expression in the bladder
urothelium and lamina propria

The manuscript in the following chapter is presented as a pre-publication copy, prior to the reviewer and publisher's requested alterations. The final version of this manuscript can be accessed online from the journal. Formatting and terminology changes have been made to maintain consistency throughout this thesis.

All data presented in this manuscript is the sole work of C. Moro

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6.1 ABSTRACT

The inner lining of the urinary bladder (urothelium/lamina propria) develops spontaneous phasic contractile activity and the aims of this study were to investigate the role of adrenoceptor subtypes in regulating this activity. The responses of isolated strips of porcine urothelium/lamina propria to noradrenaline, phenylephrine and isoprenaline were obtained in the absence and presence of receptor subtype selective antagonists. Quantitative real-time PCR was undertaken to assess the expression of adrenoceptor genes. The tissues expressed all α_1 - and β -adrenoceptor subtypes with the α_{1A} -, α_{1B} - and β_2 -adrenoceptors being the predominant receptors at the mRNA level. In functional experiments, the rate of phasic contractions and the basal tension were increased by the α_1 -adrenoceptor agonists phenylephrine (100 μ M) and A61603 (10 μ M). Rate and tension responses to phenylephrine were reduced by low concentrations of tamsulosin (3 nM) and RS100329 (10 nM), but were unaffected by BMY7378 (100 nM), prazosin (10 nM) and RS17053 (1 μ M). In contrast, isoprenaline and salbutamol (both 1 μ M) induced a relaxation of tissues and slowing of phasic contractions. Rate and tension responses to isoprenaline were inhibited by propranolol (100 nM) or a combination of CGP20712A (30 nM) and ICI118551 (70 nM), while the rate responses were also significantly inhibited by ICI118551 alone (70 nM). All α_1 - and β -adrenoceptor subtypes are expressed in the pig urothelium/lamina propria, but the $\alpha_{1A/L}$ -adrenoceptor appears to mediate increases in contractile rate and tension. The β -adrenoceptor induced inhibition of spontaneous contractile activity appears to be predominately mediated by β_2 -adrenoceptors with possibly β_1 - and β_2 -adrenoceptors involved in the tension responses.

6.2 INTRODUCTION

The functions of the bladder include both relaxation, which occurs during bladder filling, and contraction which occurs during the emptying phase of the micturition cycle. Detrusor smooth muscle contraction to muscarinic agonists is mediated via M₃ muscarinic receptors (Chess-Williams, 2002a), while noradrenaline relaxes the tissue via β -adrenoceptors, although the subtype involved appears to be species dependent (reviewed by Michel et al., 2006). α -adrenoceptors are also present on the detrusor muscle, but receptor density is low and responses to α -adrenoceptor agonists are small or non-existent (Michel and Vrydag, 2006).

Over recent years the importance of the inner epithelial lining of the bladder, the urothelium, and the underlying lamina propria in regulating bladder function has been increasingly recognised. The lamina propria, is the layer of tissue between the inner epithelial layer of the bladder wall and the underlying detrusor smooth muscle. This inner region of the bladder wall has a barrier function, but also when stretched it releases a number of factors that influence detrusor contraction and afferent nerve sensitivity (Birder, 2010). In addition, the urothelium with lamina propria from the dome of the pig bladder (Moro et al., 2012; Sadananda et al., 2008) and also strips of lamina propria from the rabbit urethra (Mattiasson et al., 1985b, Zygmunt et al., 1993) have been shown to contract to agonists such as carbachol, neurokinin-A and noradrenaline. Isolated strips of urothelium with lamina propria also develop spontaneous contractile activity, with carbachol and also stretch-induced release of acetylcholine mediating increases in spontaneous contractile frequency via M₃ receptors (Moro et al., 2011).

The lamina propria of the human bladder has been shown to be immunoreactive for tyrosine hydroxylase (Dixon et al., 1999, Jen et al., 1995) indicating a sympathetic innervations, and the lamina propria of the rabbit urethra contracts in response to phenylephrine demonstrating that α_1 -adrenoceptors are present (Mattiasson et al., 1985). Three α_1 -adrenoceptor subtypes have been identified at the molecular and functional level (α_{1A} , α_{1B} , α_{1D} ; Alexander et al., 2011) and another, termed the α_{1L} -adrenoceptor because of its low affinity for prazosin, has been recognised in functional experiments (Flavahan and Vanhoutte, 1986). Which α_1 -adrenoceptor subtypes are present in porcine urothelium and lamina propria and their functional role has not been investigated. Similarly, all three β -adrenoceptor subtypes are expressed in the human lamina propria at the mRNA and protein level (Otsuka et al., 2008), but their influence on contractile activity is unknown. Many studies have identified the pig bladder as a suitable model for human bladder pharmacology (Crowe and Burnstock, 1989, Goepel et al., 1997, Sibley, 1984). The present study examines which α - and β -adrenoceptors are expressed in the pig urinary bladder urothelium and lamina propria, and identifies the adrenoceptor subtypes responsible for mediating the frequency and tension responses of this tissue.

6.3 MATERIALS AND METHODS

6.3.1 Functional organ bath studies

Fresh bladders from Large White-Landrace pigs (6 months old, ~80 kg) were obtained from a local abattoir and immediately immersed in cold Krebs-bicarbonate solution (composition in mM: NaCl 188.4, NaHCO₃ 24.9, CaCl₂ 1.9, MgSO₄ 1.15, KCL 4.7, KH₂PO₄ 1.15 and D-glucose 11.7). The bladders were opened longitudinally and full thickness strips of anterior

wall from the dome region were removed. From these tissues, strips of urothelium with lamina propria were prepared (20 x 5 mm) and immersed in Krebs-bicarbonate solution, maintained at 37°C and gassed with 5% CO₂ in oxygen. The tissues were attached to isometric force transducers (ADInstruments MCT050/D) and tensions recorded with a Powerlab system using Labchart v7 software (ADInstruments, Castle Hill, Australia). After washing with fresh Krebs solution the tissues were allowed to equilibrate for 45 minutes under a baseline tension of ~2g before starting drug additions. For studies with antagonists, adjacent pieces of tissue were dissected, set up in pairs under identical conditions and allowed to equilibrate. One tissue was incubated with the antagonist for 30 minutes, and the other remained as a control in the absence of any antagonist. Where antagonists selective for a particular receptor subtype have been used, affinity values were obtained from the literature and the concentration carefully selected to give a maximum antagonism of the receptor without an action on the other receptor subtypes.

6.3.2 RT-PCR studies

Strips of urothelium with lamina propria (n = 6) were finely dissected with scissors under magnification and immediately immersed in RNALater solution (Ambion Inc.). The urothelium and lamina propria (~0.2g each) was then homogenised and RNA extracted with a Trizol *plus* RNA purification kit (Invitrogen Cat No. 12183-555) as per manufacturer's instructions. All storage of RNA was maintained at -80°C and experiments performed within 3 months of the initial extraction. A NanoDrop 1000 Spectrophotometer (Thermo Scientific) was used to measure the total RNA quantity (ng/μL) and to assess the purity (260/280 ratio). The integrity of the RNA was also performed for all extractions with formaldehyde agarose gel electrophoresis in ethidium bromide staining for 28s and 18s RNA band visualisation.

cDNA synthesis was then performed using SuperScript III First-Strand Synthesis SuperMix from the qRT-PCR kit (Invitrogen). Initial incubation was in a reaction mix (10 µL of 2X RT reaction mix; 2µL of reverse transcriptase enzyme mix; 8 µL of eluted RNA) and performed at 25°C for 10 minutes.

An iQTM SYBR Green Super Mix (BioRad) was used to amplify the cDNA targeted genes. Real-time PCR was performed with a Research Rotor-Gene 3000 (Corbett). 5 µL of cDNA was used to amplify the genes. PCR for ADRB3 was undertaken in triplicate with the following conditions: 94°C: 10 min (x1), Cycle 2, 94°C: 30s, 51°C: 15sec, 72°C: 15sec (x40). All other genes (Table 10) were investigated with the following conditions: cycle 1, 94°C 10 min (x1), Cycle 2, 94°C: 30s, 60°C: 30s, 72°C: 30s (x45). A post-PCR amplification protocol was preset before the run to obtain melt curve representations, ramping from 50 to 99°C with 1°C increases every 5 seconds. The expression levels of each target gene were expressed relative to the PCR amplification of the housekeeping gene β -actin.

Gene	Forward	Reverse	Amplicon Size
α_{1A}	CCATTGGGTCTTTCTTTCCT	TTTAAACTCTTGACTGGAGCAC	124
α_{1B}	GGTCATTCTGGTCATGTACTG	TCGTGAAAGTTCTTGGAGTG	134
α_{1D}	TTTCTTCGTCCTACCACTG	GTTGAAGTAGCCCAGCC	90
$\beta 1$	CCAACCTCTTCATCATGTCC	CACAGTACGTCCACCGA	144
$\beta 2$	GATCCATGACAACCTCATCC	GAAGGCCATCCTGAAATCTG	117
$\beta 3$	AGACTCCAGACCATGACC	ACAGCTCGCAACAAGTG	132
β -actin	ATGGGCCAGAAGGACTC	GCAGCTCGTTGTAGAAGG	144

Table 10: Porcine receptor-specific PCR primer sequence and PCR amplicon sizes.

6.3.3 *Data analysis and statistical procedures*

Measurements of frequency and baseline tension were taken at the peak response after the addition of each agonist. The frequency of contractions was expressed as the number of phasic waves per minute (cycles min^{-1}) and the baseline tension as grams (g). The baseline tension was taken as the lowest point of the spontaneous phasic contractions. Mean \pm standard error of mean (SEM) values in the absence and presence of drugs were compared using a Student's two-tailed *t*-test with $P < 0.05$ being taken as statistically significant. Prism software (GraphPad v4, San Diego, CA, USA) was used for statistical analysis of data. A one-way analysis of variance (ANOVA) with a Dunnett post test was applied for analysis of more than one sample (Table 11). *n* represents the number of different porcine tissues used in the study. Real-time PCR data were calculated using the cycle threshold determination method (Corbett rotor gene 6000 series software v1.7).

6.3.4 *Drugs, chemical reagents and other materials*

(\pm)-Noradrenaline hydrogen tartrate, CGP20712A methanesulfonate salt, clonidine hydrochloride, isoprenaline hydrochloride, phentolamine hydrochloride, (R)-(-)-phenylephrine hydrochloride, and prazosin hydrochloride were purchased from Sigma (St Louis, Missouri, USA). Tamsulosin was a gift provided by Astellas Pharma (Leiderdorp, NL). A61603 hydrobromide, BMY7378 dihydrochloride, BRL37344 sodium salt, CL316243 disodium salt, ICI118551 hydrochloride, (\pm)-propranolol hydrochloride, RS17053 hydrochloride, RS100329 hydrochloride, SR59230A, salbutamol sulphate, and UK14,304 were purchased from Tocris (Ellisville, Missouri, USA). Oligonucleotide primers (Table 10) were obtained from Geneworks (Thebarton, South Australia).

6.4 RESULTS

6.4.1 *Spontaneous phasic contractions*

The urothelium and lamina propria exhibited spontaneous contractions within 10 minutes of being placed in the organ bath (Figure 6-1). This regular phasic activity of the urothelium was present throughout the course of the experiment and occurred at a spontaneous contractile frequency of 3.48 ± 0.07 cycles min^{-1} and an amplitude of $0.68 \pm 0.03\text{g}$ ($n = 211$).

6.4.2 *Responses to noradrenaline*

Noradrenaline ($10 \mu\text{M}$, $n = 8$) significantly reduced the frequency of spontaneous contractions by $15.2 \pm 6.5\%$ and baseline tension by $22 \pm 4\%$ (Table 11). The frequency responses were significantly enhanced in the presence of phentolamine ($10 \mu\text{M}$), noradrenaline then reducing the frequency of contractions by $39 \pm 7\%$, while relaxation of the tissues to noradrenaline was not significantly affected (Table 11). In contrast, in the presence of propranolol ($1 \mu\text{M}$), responses to noradrenaline ($10 \mu\text{M}$, $n = 8$) were converted to increases in the rate of spontaneous contractions ($41 \pm 7\%$ increase) and the baseline tension increased by $52 \pm 10\%$ (Table 11).

Table 11: Frequency and tension responses of urothelium/lamina propria to noradrenaline.

Drug	Change in frequency (cycles min ⁻¹)	Change in tension (grams)	n
Noradrenaline (10 µM)	-0.57 ± 0.24	-0.64 ± 0.16	8
Noradrenaline (10 µM) + Phentolamine (10 µM)	-1.40 ± 0.25 ^a	-0.82 ± 0.06	8
Noradrenaline (10 µM) + Propanolol (1 µM)	1.34 ± 0.20 ^b	0.76 ± 0.13 ^b	8

Values are mean ± SEM.

^aSignificantly different from noradrenaline alone, $P < 0.05$ (ANOVA)

^bSignificantly different from noradrenaline alone, $P < 0.01$ (ANOVA)

6.4.3 α -adrenoceptor responses

The α_2 -adrenoceptor agonists clonidine and UK14,304 are highly potent agonists at α_2 -adrenoceptors and produce responses at nano-molar concentrations if these receptors are present in a tissue. However at concentrations up to 1µM, neither agonist had any effect on either the spontaneous contractile frequency or the basal tension developed by tissues (n=8). Higher concentration of these drugs will activate α_1 -adrenoceptors and thus were not examined. In contrast, the α_1 -adrenoceptor selective agonist phenylephrine (100 µM) increased the spontaneous activity by $41 \pm 8\%$ and the baseline tension by $22 \pm 5\%$ (n = 21, $P < 0.001$ for both). At a 10-fold lower concentration, the α_{1A} -adrenoceptor selective agonist A61603 (10 µM) produced a similar increase in spontaneous contractile frequency $31 \pm 4\%$ and also increased baseline tension by $31 \pm 4\%$ (n = 26, $P < 0.001$ for both; Figure 6-1). Lower concentrations of phenylephrine (1 – 10 µM, n = 8) did not cause any significant responses for frequency or tension, yet lower concentrations of A61603 (3 µM, n = 8, data not shown) resulted in significant increases for frequency by $27 \pm 9\%$ ($P < 0.05$) and baseline tension by $18 \pm 5\%$ ($P < 0.01$).

The increase in the frequency of contractions produced by phenylephrine was significantly reduced by low concentrations of the α_{1A} -adrenoceptor antagonists, RS100329 (10 nM, n = 8) and tamsulosin (3 nM, n = 8), but not by the α_{1D} -adrenoceptor selective antagonist BMY7378 (100 nM, n = 8; Figure 6-2). Increases in basal tension induced by phenylephrine (100 μ M) were similarly reduced by RS100329 (10 nM, n = 8, $P < 0.05$) and tamsulosin (3 nM, n = 8, $P < 0.05$), but not BMY7378 (100 nM, n = 8, Figure 6-2). Two antagonists that discriminate between α_{1A} - and α_{1L} -adrenoceptors, prazosin and RS17053 were also examined. Neither prazosin (10 nM, n = 10) nor RS17053 (1 μ M, n = 8) had any significant effect on either frequency or tension responses to phenylephrine (Figure 6-2).

6.4.4 β -adrenoceptor responses

Isoprenaline (1 μ M, n = 62) reduced the spontaneous contractile frequency of tissue strips by $34 \pm 2\%$ (1.21 ± 0.08 cycles min^{-1} , $P < 0.001$) and the baseline tension by $37 \pm 2\%$ ($0.66 \pm 0.44\text{g}$, $P < 0.001$, Figure 6-1). A higher concentration of isoprenaline (10 μ M, n = 16) produced a greater inhibition of the contractile frequency ($49 \pm 1\%$, $P < 0.01$), but the depression of baseline tension was similar to that obtained with the lower concentration of isoprenaline ($40 \pm 3\%$). Salbutamol (1 μ M, n = 7) reduced the frequency of spontaneous contractions by $33 \pm 6\%$ and the baseline tension by $22 \pm 2\%$ ($P < 0.001$ for both responses, Figure 6-1). A higher concentration of salbutamol (10 μ M) did not induce greater inhibition of the tissues. The β_3 -adrenoceptor selective agonist, BRL37344, at concentrations up to 10 μ M (n = 8, data not shown), did not affect the frequency of spontaneous contractions or baseline tension. Another β_3 -adrenoceptor selective agonist, CL316243, was similarly without effect over a range of concentrations up to 1 μ M.

Isoprenaline (1 μ M; Figure 6-3) induced relaxation and a slowing of spontaneous phasic contractions and both these responses were significantly reduced in the presence of propranolol (100 nM, n = 8) or a combination of the β_1 -adrenoceptor antagonist CGP20712A (30 nM, n = 8) and β_2 -adrenoceptor antagonist ICI118551 (70 nM, n = 11). However the responses were unaffected by CGP20712A (β_1 -adrenoceptor antagonist, n = 8), or SR59230A (β_3 -adrenoceptor antagonist, n = 8). The β_2 -adrenoceptor antagonist ICI118551 (70 nM, n = 12) significantly reduced frequency responses to isoprenaline ($P < 0.05$), but not relaxation responses to this agonist (Figure 6-3).

6.4.5 Real-Time Polymerase Chain Reaction

RNA was extracted from isolated urothelium and lamina propria samples (n = 6, run in triplicate) and tested for integrity prior to being converted into cDNA. RNA concentrations and 260/280 ratios were as follows: Sample 1: 602 ng/ μ L, 2.06; Sample 2: 699ng/ μ L, 2.10; Sample 3: 1050 ng/ μ L, 2.08; Sample 4: 620 ng/ μ L 2.09, Sample 5: 823 ng/ μ L; 2.10, Sample 6: 590 ng/ μ L, 2.11. The real-time PCR products for all six adrenoceptor genes and the housekeeping gene β -actin were run in triplicate and the end product visualised on 2% agarose gels to identify that all genes demonstrated the correct fragment sizes (Figure 6-4). For $\alpha 1$ -adrenoceptors, the greatest expression was observed for the $\alpha 1A$ - and $\alpha 1B$ -adrenoceptor subtypes, while that for the $\alpha 1D$ -adrenoceptor was only half that obtained for $\alpha 1A$ - and $\alpha 1B$ -adrenoceptors (Figure 6-4). All three β -adrenoceptor subtypes were expressed in these tissues. The expression was highest for $\beta 2$ -adrenoceptors; which was nearly double that for either $\beta 1$ - or $\beta 3$ -adrenoceptors (Figure 6 4).

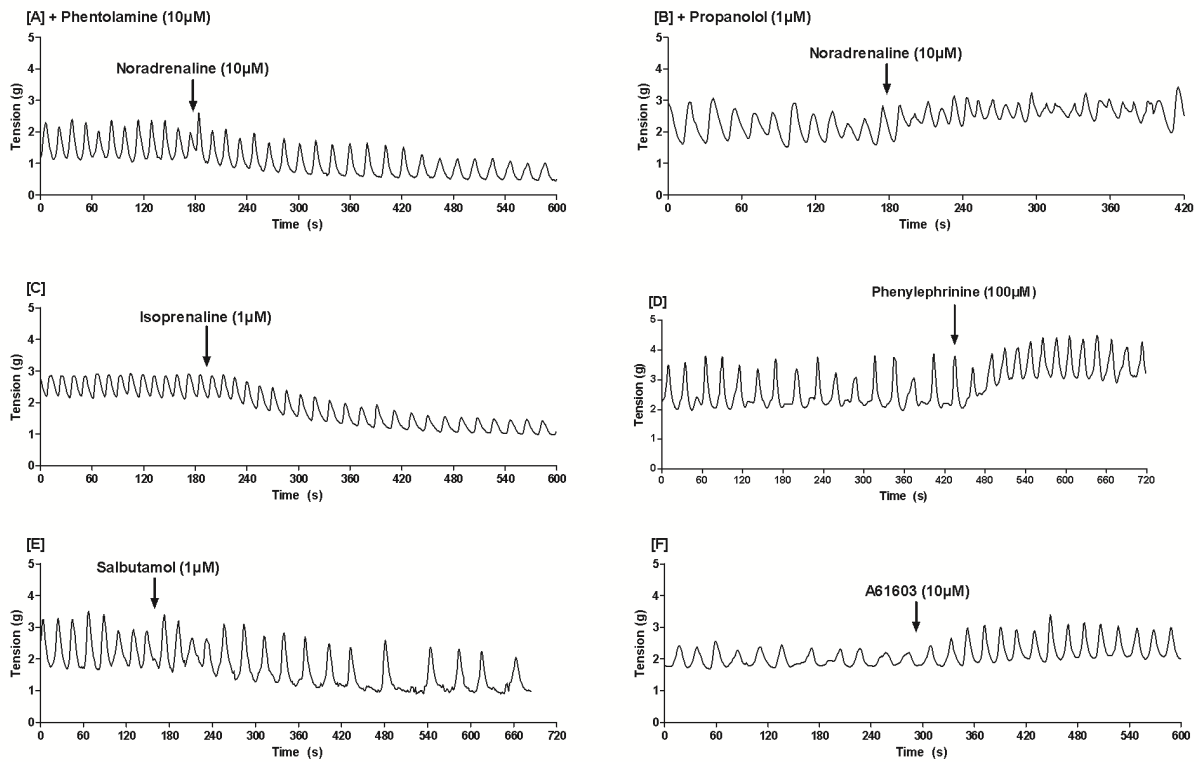


Figure 6-1: Typical experimental traces showing the effects of adrenoceptor agonists on urothelium and lamina propria contractile activity. [A] Noradrenaline (10 μ M) in the presence of phentolamine (10 μ M), [B] noradrenaline (10 μ M) in the presence of propanolol (1 μ M), [C] isoprenaline (1 μ M), [D] phenylephrine (100 μ M), [E] salbutamol (1 μ M) and [F] A61603 (10 μ M).

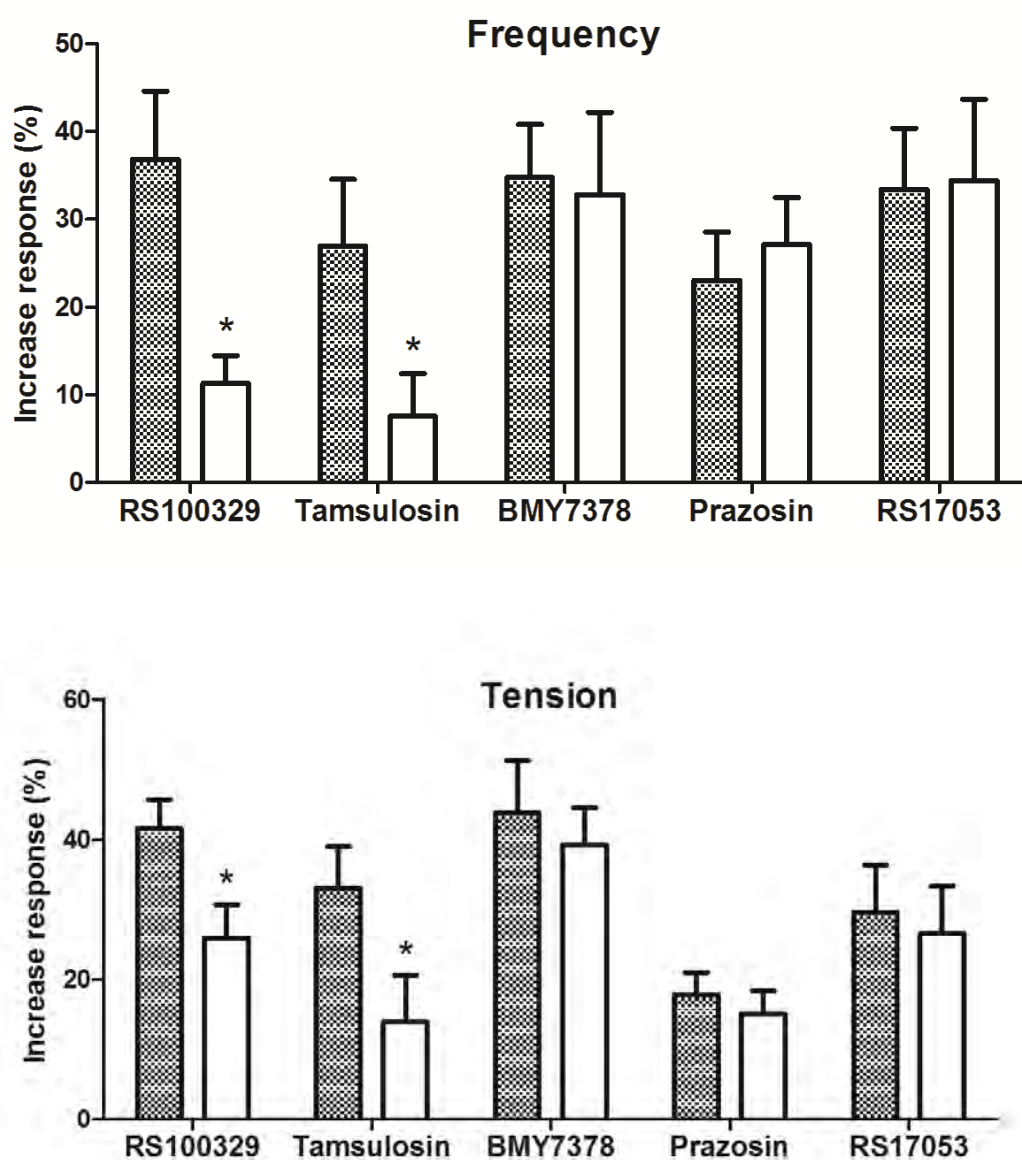


Figure 6-2: Frequency and tension responses of urothelium/lamina propria strips induced by phenylephrine (100μM), in the absence (shaded columns) and in the presence (open columns) of antagonists: RS100329 (10nM, n=8), tamsulosin (3nM, n=8), BMY7378 (100nM, n=8), prazosin (10nM, n=10) and RS17053 (1μM, n=8). Columns represent the mean ± SEM. Student's two-tailed *t*-test: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

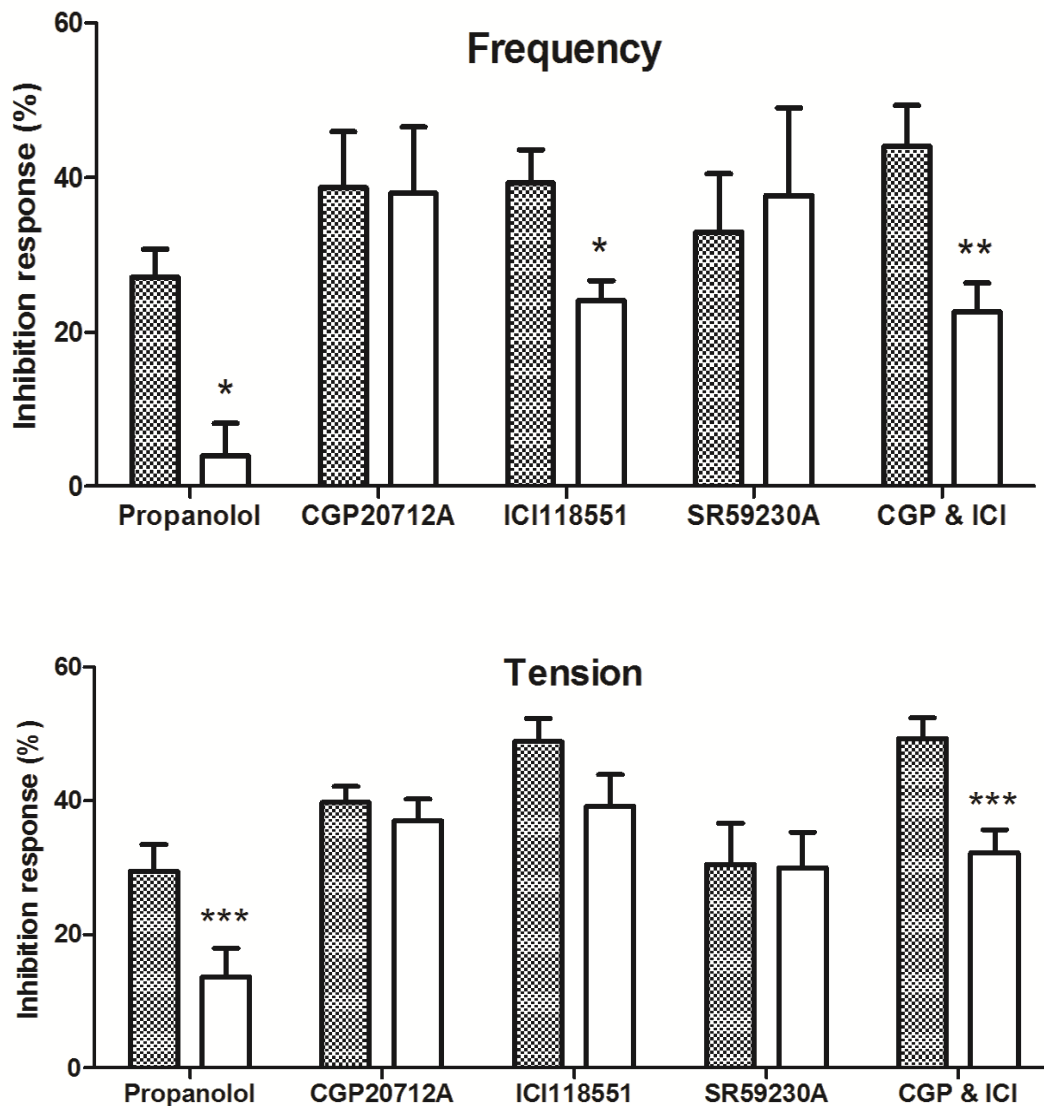


Figure 6-3: Frequency and tension inhibitions induced by isoprenaline in the absence (shaded columns) and presence (open columns) of antagonists: propanolol (100nM, n=8), CGP20712A (30nM, n=8), ICI118551 (70nM, n=8), SR59230A (30nM, n=8), and a combination of ICI118551 (70nM) & CGP20721A (30nM, n=11, CGP & ICI). Responses are expressed as the percentage increase (upper figure) or inhibition (lower figure) of rate and tension. Columns represent the mean \pm SEM. Student's two-tailed *t*-test: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

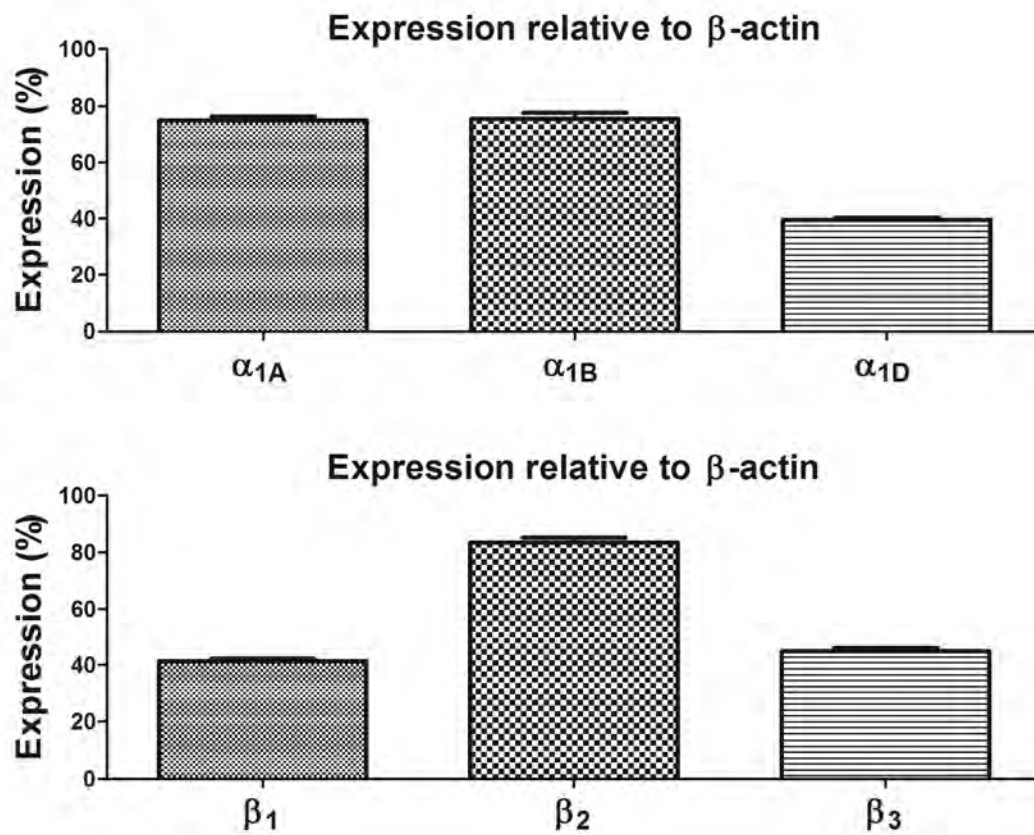


Figure 6-4: Relative expression of each adrenoceptor gene. Expression values are based relative to β -actin expression for the α -adrenoceptors (upper panel) and β -adrenoceptors (middle panel) and expressed as the mean \pm SEM (n=8 for each).

6.5 DISCUSSION

In the absence of any exogenous agonist, the urothelium with lamina propria samples developed spontaneous phasic contractions. Initial data obtained with noradrenaline suggests that both α - and β -adrenoceptors are present and functional in this tissue: α -adrenoceptors mediating contraction and increases in frequency, whilst β -adrenoceptor activation relaxes the tissue and slows the frequency of phasic contractions. The dominant response to the physiological agonist noradrenaline is β -adrenoceptor mediated relaxation and slowing, since these were the responses obtained to noradrenaline in the absence of antagonists. However, these inhibitory responses to noradrenaline were enhanced in the presence of phentolamine, indicating that α -adrenoceptors were reducing these inhibitory responses to noradrenaline in the absence of any antagonists. Thus both α - and β -adrenoceptors are involved in mediating responses to the endogenous agonist noradrenaline.

6.5.1 α -adrenoceptors

The α -adrenoceptor mediated responses of the urothelium and lamina propria appear to involve the α_1 -adrenoceptor subtype since both phenylephrine and A61603 increased phasic frequency, whilst the agonists with predominantly α_2 -adrenoceptor agonist selectivity, clonidine and UK14304, had no effect. The tissue expresses mRNA for all three cloned α_1 -adrenoceptors, but the levels of α_{1A} and α_{1B} -adrenoceptor mRNA are greater than that for the α_{1D} -adrenoceptor subtype. On isolated tissues, both phenylephrine and the α_{1A} -adrenoceptor selective agonist A61603 (Knepper et al., 1995) induced increases in baseline tension and the rate of phasic contractions. The tension and rate responses for A61603 (10 μ M) were significantly greater than those to the same concentration of phenylephrine, due to the higher

affinity of A61603 at the α_{1A} -adrenoceptor subtype. Higher concentrations of phenylephrine (100 μ M) produced a greater response almost identical to that produced by the 10-fold lower concentration of A61603. These agonist data suggest that responses were mediated via the α_{1A} -adrenoceptor subtype.

The antagonist data support these findings. Responses to phenylephrine were reduced by a low concentration of tamsulosin (3 nM), an antagonist possessing high affinity for α_{1A} - and α_{1D} -adrenoceptors (Noble et al., 1997). Thus responses were not mediated via α_{1B} -adrenoceptors. In addition, rate and tension responses to phenylephrine were potently inhibited by RS100329, a selective antagonist for the α_{1A} -adrenoceptor subtype. RS100329 has a pK_i at α_{1A} -adrenoceptors of 9.6 and demonstrates a 126- and 50-fold selectivity over α_{1B} and α_{1D} -adrenoceptor subtypes (Conley et al., 2001; Williams et al., 1999), thus at the concentration used in the present study (10 nM) it would only have an action at α_{1A} -adrenoceptors. The lack of involvement of α_{1D} -adrenoceptors was confirmed using BMY7378, an antagonist with a greater than 100-fold high affinity for the α_{1D} -adrenoceptor subtype over α_{1A} and α_{1B} -adrenoceptors (Piascik et al., 1995, Yang and Endoh, 1997). The lack of effect at a concentration of 100 nM with BMY7378 supports the conclusion that α_{1D} -adrenoceptors are not involved in mediating responses of the urothelium and lamina propria, and that the α_{1A} -adrenoceptor is the functional α_1 -adrenoceptor in this tissue.

A number of studies have demonstrated that contractions of the bladder neck and prostate are mediated via α_1 -adrenoceptors that, unlike the cloned receptors, have a low affinity for the antagonist prazosin and this putative receptor subtype has been termed the α_{1L} -adrenoceptor or $\alpha_{1A/L}$ -adrenoceptor (Alexander et al., 2011). It is generally accepted that this functional receptor exists as a conformational state of the α_{1A} -adrenoceptor, but the mechanisms

involved are unclear and it has been suggested that experimental conditions (Ford et al., 1996, Ford et al., 1997) or specific intracellular proteins (Nishimune et al., 2010) may be involved. Both prazosin and RS17053 have a low affinity for this form of the receptor compared to the α_{1A} -adrenoceptor, and the lack of effect for these antagonists in the present study suggests that it is this conformational state ($\alpha_{A/L}$ -adrenoceptor) that mediates contraction of this tissue.

6.5.2 β -adrenoceptors

Noradrenaline, isoprenaline and salbutamol all induced relaxation of tissues and a slowing of the frequency of phasic contractions. Propranolol antagonised the inhibitory effects of isoprenaline and abolished those to noradrenaline, converting them to a small contraction and an increase in phasic rate of contractions to noradrenaline. All three subtypes of β -adrenoceptor (β_1 , β_2 and β_3) were present at the mRNA level, with the expression of β_2 -adrenoceptors being double that of either the β_1 - or β_3 -adrenoceptor subtypes. Isoprenaline reduced the rate of phasic contractions and these inhibitory responses were reduced when β_2 -adrenoceptors were antagonised with either propranolol (β_1 - and β_2 - antagonist), ICI118551 (β_2 -antagonist) or a combination of ICI118551 and CGP20712A (β_1 - antagonist). The responses were insensitive to CGP20712A alone or SR59230A (β_3 - antagonist) indicating β_2 -adrenoceptors are the predominant functional β -adrenoceptor subtype inhibiting spontaneous contractile frequency in these tissues. For relaxation responses, combined β_1 - and β_2 -adrenoceptor antagonism appeared to be required to significantly inhibit responses to isoprenaline and CGP20712A alone, ICI118551 alone or SR59230A alone failed to influence responses. In some species, the responses of detrusor smooth muscle to isoprenaline are mediated via β_3 -adrenoceptors, but for porcine urothelium and lamina propria, selective

concentrations of SR59230A did not affect frequency or tension responses to isoprenaline, and also CL316243 and BRL37344, two selective β_3 -adrenoceptor agonists did not influence either response. Thus inhibitory responses of the urothelium and lamina propria to isoprenaline appear to be mediated predominantly by β_2 -adrenoceptors with a possible contribution from β_1 -adrenoceptors for tension responses.

The mechanisms involved in these responses of the urothelium and lamina propria are not clear. Tension responses to neurokinin-A and carbachol are present after fine dissection and removal of smooth muscle from the preparations and it has been suggested that myfibroblasts may be involved (Moro et al., 2011; Sadananda et al., 2009). In addition, spontaneously generated electrophysiological events have been observed in interstitial cells located within the lamina propria (Fry et al., 2007). The function of this contractile activity is also uncertain. It may act to ensure folding of the urothelium in the empty bladder or alternatively it may act to enhance sensory nerve function (Kanai et al., 2011). It has been reported that spontaneous calcium and electrical activity arise in the urothelium-lamina propria interface and pass into the detrusor regions (Kanai et al., 2007) and this is increased in tissues from cats with interstitial cystitis (Ikeda et al., 2009). Therefore, in situations where electrical coupling is enhanced, it may be possible that the lamina propria may drive larger spontaneous contractions of the detrusor smooth muscle.

6.6 CONCLUSIONS

In conclusion, the urothelium and lamina propria of the pig bladder develops spontaneous contractile activity that can be regulated by the sympathetic nervous system. The force of contraction and the frequency of spontaneous contractions are increased by α_1 -adrenoceptor stimulation and depressed by β -adrenoceptor stimulation. The predominant α_1 -adrenoceptor subtype has the pharmacological characteristics of the $\alpha_{1A/L}$ -adrenoceptor subtype, while β_2 -adrenoceptors (with possibly a β_1 - contribution for tension) appear to be the predominant receptors mediating inhibitory responses.

Chapter 7

Non-adrenergic, non-cholinergic, non-purinergic contractions of the urothelium/lamina propria of the pig bladder

The manuscript in the following chapter is presented as a pre-publication copy, prior to the reviewer and publisher's requested alterations. The final version of this manuscript can be accessed online from the journal. Formatting and terminology changes have been made to maintain consistency throughout this thesis.

All data presented in this manuscript is the sole work of C. Moro

Published manuscript

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Moro C, Chess Williams R (2009). Urothelial contractions to electrical field stimulation are mediated by an unidentified neurotransmitter(s). In: Abstracts from the 39th annual meeting of the International Continence Society Vol. 28, 2009 edn. San Francisco, USA.

7.1 ABSTRACT

Objectives: Acetylcholine, and to a lesser extent ATP, mediate neurogenic contractions of bladder smooth muscle. Recently, the urothelium and lamina propria has also been shown to have contractile properties, but the neurotransmitters involved in mediating responses to nerve stimulation have not been investigated.

Methods: Isolated strips of porcine urothelium with lamina propria were electrically field stimulated and contractions recorded. Drugs interfering with neurotransmission were then employed to identify which neurotransmitters mediated responses.

Results: Strips of urothelium/lamina propria developed spontaneous contractions with a frequency of 3.50 ± 0.10 cycles min^{-1} and amplitude of $0.84 \pm 0.06\text{g}$. Electrical field stimulation at 5, 10, and 20 Hz resulted in frequency-related contractions ($1.13 \pm 0.36\text{g}$, $1.59 \pm 0.46\text{g}$ and $2.20 \pm 0.53\text{g}$ respectively, $n = 13$) and these were reduced in the presence of tetrodotoxin ($1 \mu\text{M}$) by $77 \pm 20\%$ at 5 Hz, $79 \pm 7\%$ at 10 Hz and $74 \pm 12\%$ at 20 Hz (all $P < 0.01$) indicating they were predominantly neurogenic in nature. Neither the muscarinic antagonist atropine ($10 \mu\text{M}$), the adrenergic neurone blocker guanethidine ($10 \mu\text{M}$), nor desensitisation of the purinergic receptors with α,β -methylene ATP ($10 \mu\text{M}$) affected the contractile amplitude. Similarly, responses were not affected by the nitric oxide synthase inhibitor L-NNA ($100 \mu\text{M}$) or drugs that interfere with peptide neurotransmission (capsaicin, NK2 antagonist GR159897, protease inhibitors).

Conclusions: Electrical depolarisation of the nerves present in the porcine urothelium/lamina propria results in frequency-dependent contractions which are predominantly neurogenic in nature. These contractions are resistant to drugs which inhibit the adrenergic, cholinergic and purinergic systems. The neurotransmitter involved in the responses of this tissue is therefore unknown but does not appear to be a peptide.

7.2 INTRODUCTION

The detrusor smooth muscle of the urinary bladder receives both sympathetic and parasympathetic innervation. During bladder filling, activity in the sympathetic nerves results in detrusor relaxation via stimulation of β -adrenoceptors, thus allowing an increase in bladder volume without increases in intravesical pressure. The β -adrenoceptor subtype involved is species dependent but responses are mediated via the β_3 -adrenoceptor subtype in pig (Yamanishi et al., 2002b) and human (Igawa et al., 1999, Yamaguchi, 2002, Badawi et al., 2007) detrusor muscle. During bladder emptying, activity in the parasympathetic nerves results in detrusor contraction which is mediated via M_3 muscarinic receptors (Sellers et al., 2000b, Chess-Williams et al., 2001).

Detrusor responses to nerve stimulation during electrical field stimulation (EFS) may also involve a non-cholinergic, non-adrenergic (NANC) neurotransmitter that was first identified as ATP by Burnstock *et al.* (1972). NANC responses have been observed in the bladders of several species (Sibley, 1984, Somogyi et al., 1998), although in human this component is usually only observed in the diseased bladder (Sjogren et al., 1982).

It has been reported that the inner lining of the bladder and urethra, the urothelium and lamina propria, exhibits contractile properties (Sadananda et al., 2008, Moro et al., 2011, Mattiasson et al., 1985a). The porcine tissue also exhibits spontaneous phasic contractile activity and contracts in response to neurokinin (Sadananda et al., 2008) and muscarinic (Moro et al., 2011) receptor stimulation. Nitric oxide (NO) donors relax this tissue, although there does not appear to be endogenous release of nitric oxide (Moro et al., 2012).

The responses of this tissue to electrical field stimulation have never been investigated and the aim of this study was to assess the relative involvement of the neurotransmitters acetylcholine, noradrenaline, ATP and nitric oxide in mediating neurogenic responses of the urothelium and lamina propria.

7.3 MATERIALS AND METHODS

Bladders from pigs (6 months age, ~70 Kg) were obtained from a local abattoir and immediately immersed in Krebs-bicarbonate solution composed of NaCl (188.4 mM), NaHCO₃ (24.9 mM), CaCl₂ (1.9 mM), MgSO₄ (1.2 mM), KH₂PO₄ (1.2 mM) and glucose (11.7 mM). The bladder was opened longitudinally and strips of urothelium plus lamina propria (20 x 5 mm) were dissected from the anterior wall of the dome of the bladder. These were set up under 2g tension in 10 mL baths containing warmed Krebs-bicarbonate solution at 37°C and gassed with 5% CO₂ in oxygen. Isometric tension developed by tissues was recorded via a Powerlab system using Labchart software (AdInstruments, Castle Hill, Australia).

Preliminary experiments indicated that the optimal stimulation parameters for tissue strips was 5 second trains of pulses (0.5 ms duration, 20 V) applied every 100 seconds. Three stimulation frequencies (5, 10 and 20 Hz) were examined and the neurotoxin tetrodotoxin (1 µM) was used to confirm the neurogenic origin of contractions. Responses to electrical field stimulation (EFS) were measured as the increase in developed tension above the baseline spontaneous contractions. Control experiments were performed without the addition of any drugs and these established that responses to EFS were stable and reproducible over the time

course of the experiment. Data obtained at each stimulation frequency in the absence and presence of drugs were analysed using paired Student's *t*-tests.

7.3.1 *Drugs and solutions*

Tetrodotoxin, atropine sulfate, α,β -methyleneadenosine 5'-triphosphate lithium salt, N ω -nitro-L-arginine (L-NNA), guanethidine monosulfate, protease inhibitor cocktail (P1860, containing a mixture of protease inhibitors with broad specificity for the inhibition of serine, cysteine, aspartic and aminopeptidases), phosphoramidon disodium and capsaicin, were purchased from Sigma-Aldrich Co. (St. Louis, MO). GR159897 and neurokinin-A was purchased from Tocris Bioscience (Minneapolis, MN).

7.4 RESULTS

Strips of urothelium with lamina propria exhibited spontaneous contractile activity within 15 minutes of being set up in the organ baths. These contractions occurred with a mean amplitude of 0.84 ± 0.06 g and a frequency of 3.50 ± 0.10 cycles min^{-1} ($n = 146$). When stimulated electrically at 5 Hz, strips contracted by 1.13 ± 0.36 g above the baseline spontaneous contractions ($n = 13$, $P < 0.01$). Increasing the frequency of stimulation to 10 Hz increased the contractions to 1.59 ± 0.46 g ($P < 0.01$), while stimulation at 20 Hz resulted in contractions of 2.20 ± 0.53 g ($P < 0.001$). In the presence of tetrodotoxin (1 μM , Figure 7-1), responses were reduced by $77 \pm 20\%$ at 5 Hz, $79 \pm 7\%$ at 10 Hz, and $74 \pm 12\%$ at 20 Hz ($n = 13$, $P < 0.01$ for all frequencies) indicating that they were predominantly neurogenic in nature. The EFS contractions did not have a significant effect on the rate of spontaneous contractile activity over 20 minutes. Control experiments without the addition of any drugs demonstrated that responses to electrical field stimulation were stable over the duration of the experiment.

Atropine at concentrations up to 10 μM ($n = 8$) had no significant effect on responses of the urothelium/lamina propria strips to electrical field stimulation at any of the frequencies examined (Figure 7-2). As a positive control the effects of atropine on contractile responses of urothelium/lamina propria strips to carbachol were examined and atropine (1 μM) abolished responses to carbachol (1 μM , Figure 7-3). In further control experiments, EFS at 5 Hz induced contractions of detrusor smooth muscle strips and these were reduced by $68 \pm 8\%$ ($P < 0.05$) by atropine at a concentration of 1 μM ($n = 8$).

Similarly, neither the presence of the adrenergic neurone blocker guanethidine (10 μM , $n = 12$) nor the NO synthase inhibitor L-NNA (100 μM , $n = 15$) had any effect on the responses of the urothelium/lamina propria to field stimulation (Figure 7-2). As a positive control the actions of guanethidine (10 μM) were examined on the pig urethra stimulated at 5 Hz under identical conditions. In these control experiments guanethidine depressed contractions to field stimulation of the pig urethra by $95 \pm 3\%$ ($n = 6$).

To investigate the involvement of ATP in mediating neurogenic contractions, α,β -methylene ATP (10 μM) was used to desensitise the purinergic receptors. This agent caused a transient contraction of the tissue and subsequent responses to this agonist were reduced by $85 \pm 2\%$ ($n = 8$, $P < 0.001$, Fig 3) indicating a desensitisation of purinergic receptors. However, following this procedure, responses to field stimulation were similar to controls ($n = 8$, Fig. 2) indicating a lack of input from purinergic nerves. To further investigate a role for these classical transmitters, a cocktail of all these drugs (atropine, guanethidine and α,β -methylene ATP) was examined, but again these agents in combination failed to alter responses to electrical field stimulation of the urothelium/lamina propria tissue (Figure 7-4).

To investigate the possible involvement of peptide neurotransmitters in mediating responses, the effects of phosphoramidon (10 μ M, n = 8), and also a cocktail of protease inhibitors (Sigma P1860, 50 μ L, n = 8) were examined. None of these compounds had any effect on tissue contractions to EFS (Table 12). Similarly the addition of capsaicin (100 μ M) had no immediate effect on tissue tension or on the subsequent responses of the urothelium/lamina propria to field stimulation (n = 14, Table 12). The effect of the neurokinin-2 receptor antagonist was also examined on the contractile responses of the urothelium/lamina propria induced by EFS. Both frequency and tension were increased following administration of neurokinin-A (10 - 100 nM, $P < 0.05$, n = 8), yet at a concentration of (1 μ M, n = 6) GR159897 abolished responses to neurokinin-A (100 nM, Figure 3) but failed to have any effect on responses of these tissues to EFS.

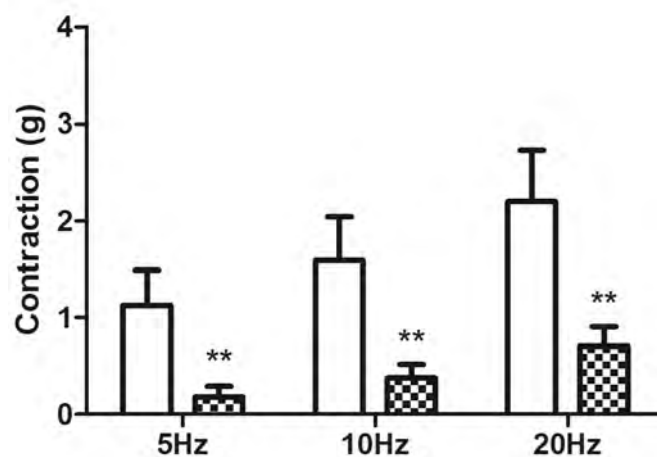


Figure 7-1: Responses of urothelium/lamina propria strips (mean \pm SEM, n = 13) to EFS at different stimulation frequencies in the absence (open columns) and presence (filled columns) of the neurotoxin tetrodotoxin (1 μ M).

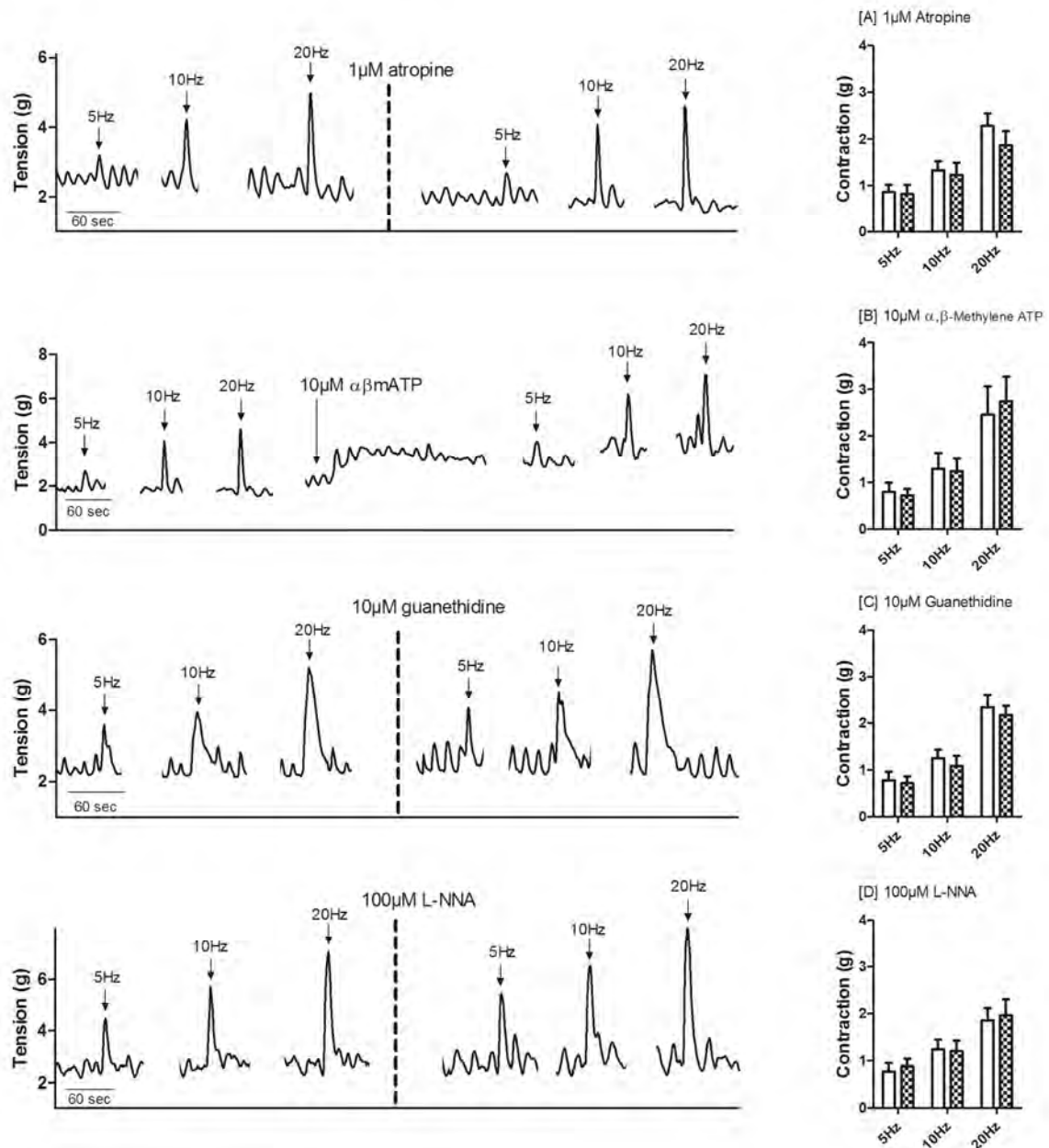


Figure 7-2: Experimental traces and histograms showing urothelium/lamina propria contractile responses to electrical field stimulation. For histograms, mean responses (\pm SEM) are shown in the absence (open columns) and presence (shaded columns) of [A] atropine (1 μ M, $n = 15$), [B] α,β -methylene ATP (10 μ M, $n = 8$), [C] guanethidine (10 μ M, $n = 12$), and [D] L-NNA (100 μ M, $n = 15$). There were no significant differences between pairs of data ($P > 0.05$, Student's paired t -test).

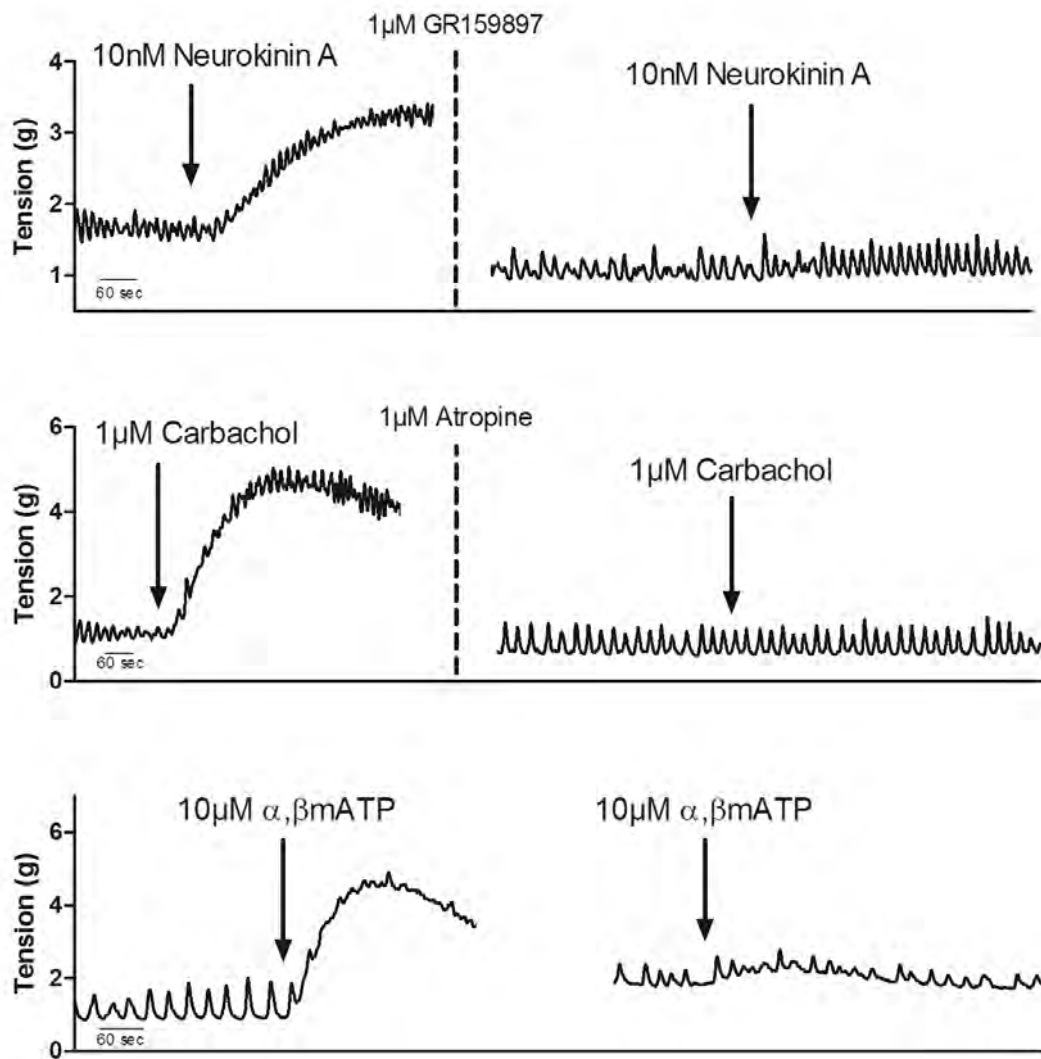


Figure 7-3: Typical experimental traces of urothelial/lamina propria contractile activity showing the effects of GR159897 on responses to neurokinin-A (top trace), atropine on responses to carbachol (middle trace) and the effects of P2X purinergic receptor desensitisation on responses to α,β -methylene ATP (lower trace).

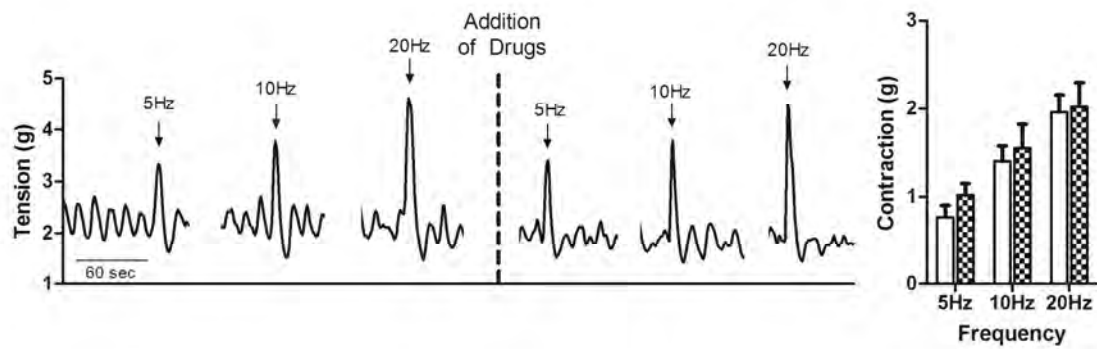


Figure 7-4: Experimental trace (left) and histogram (right) demonstrating the responses to electrical field stimulation of the urothelium/lamina propria in the absence (open columns) and in the presence (shaded columns) of a combination of atropine (1 μ M), α,β -methylene ATP (10 μ M), guanethidine (10 μ M), and L-NNA (100 μ M). This cocktail of drugs had no effect on responses (n = 16).

Table 12: Drugs influencing peptidergic neurotransmission

Urothelium		5 Hz		10 Hz		20 Hz	
Drug:	n	absence (g)	presence (g)	absence (g)	presence (g)	absence (g)	presence (g)
Phosphoramidon (10 μ M)	8	0.88 \pm 0.4	0.97 \pm 0.5	1.84 \pm 0.7	1.75 \pm 0.7	2.91 \pm 0.9	2.60 \pm 0.9
Protease Inhibitor Cocktail (50 μ L)	8	1.69 \pm 0.3	1.64 \pm 0.4	2.76 \pm 0.6	2.83 \pm 0.6	4.54 \pm 0.7	4.78 \pm 0.9
Capsaicin (100 μ M)	14	0.83 \pm 0.2	1.01 \pm 0.2	1.80 \pm 0.2	1.63 \pm 0.2	3.00 \pm 0.4	2.60 \pm 0.4
GR159897 (1 μ M)	6	0.65 \pm 0.2	0.62 \pm 0.2	0.92 \pm 0.1	0.96 \pm 0.1	1.16 \pm 0.1	1.22 \pm 0.2

Mean \pm SEM contractile responses to EFS (5, 10, 20 Hz) of urothelial/lamina propria strips in the absence and presence of drugs interfering with peptide neurotransmission: protease inhibitors (phosphoramidon & cocktail of inhibitors), depletory of sensory neuropeptides (capsaicin) and NK antagonist (GR159897). There were no significant differences between pairs of data ($P > 0.05$, Student's paired t -test).

7.5 DISCUSSION

Isolated strips of urothelium and lamina propria developed spontaneous phasic contractions that have been reported previously (2012, Moro et al., 2011). Whether this contractile activity originates in smooth muscle cells or in interstitial cells within the lamina propria is unclear (Sadananda et al., 2008, Heppner et al., 2011). Electrical field stimulation of the tissues induced contractions significantly greater than the background phasic contractions. These responses were frequency-dependent and predominantly (70 - 80%) neurogenic in origin as demonstrated by their sensitivity to the neurotoxin tetrodotoxin. Similar neurogenic responses can be observed in the detrusor smooth muscle where the majority of the contraction is mediated via acetylcholine acting on M₃ muscarinic receptors (Sellers et al., 2000b, Chess-Williams et al., 2001). However, in the urothelial/lamina propria tissue, responses were insensitive to antagonism by atropine even at concentrations up to 10 μ M. As a positive control, the actions of atropine at the lower concentration of 1 μ M were examined on isolated strips of pig detrusor muscle (stimulated under identical conditions), and in these tissues, atropine reduced contractions to electrical stimulation at 5, 10 and 20 Hz by $68 \pm 8\%$, $80 \pm 7\%$ and $88 \pm 4\%$ respectively (all $P < 0.01$, $n = 8$). Both the pig detrusor (Sellers et al., 2000b) and the urothelium/lamina propria (Moro et al., 2011) contract strongly to muscarinic agonists and these data therefore suggest that the detrusor, but not the urothelium/lamina propria, has a dominant efferent cholinergic innervation capable of inducing contraction.

Another neurotransmitter often co-released with acetylcholine is ATP and detrusor muscle expresses P2X₁ receptors that mediate a contractile response to electrical field stimulation (Somogyi et al., 1998, O'Reilly et al., 2001a, Vial and Evans, 2000). On strips of urothelium/lamina propria the potent P2X receptor agonist α,β -methylene ATP induced a

transient contraction and abolished subsequent responses to purinergic agonists, indicating desensitisation of the P2X receptors. However, responses to electrical field stimulation were not altered by this procedure suggesting that ATP does not contribute to the contractions induced by field stimulation. It has been suggested that lamina propria interstitial cells may possess P2Y receptors for ATP and respond with a rise in intracellular calcium to the P2Y receptor agonist UTP (Wu et al., 2004). However the changes in intracellular calcium were not accompanied by contractile responses in that study and in the present studies strips of urothelium/lamina propria failed to contract to UTP or UDP (100 μ M, n = 8). These results suggest that the tissue is similar to the detrusor muscle in possessing purinergic receptors (probably P2X) that can cause contraction, but unlike the detrusor, ATP does not appear to be an important neurotransmitter in the urothelium/lamina propria.

The detrusor muscle also receives a sympathetic innervation which induces relaxation via β 3-adrenoceptors in the porcine and human bladder (Yamanishi et al., 2002b, Igawa et al., 1999, Yamaguchi, 2002, Badawi et al., 2007). In the detrusor of the bladder dome, α ₁-adrenoceptor density is low and responses to α -adrenoceptor agonists are small or non-existent (Michel and Vrydag, 2006). Similarly in the urothelium/lamina propria, α ₁-adrenoceptor stimulation has only minor effects on contraction, although it has been found to increase the frequency of spontaneous contractions of this tissue (Moro et al., 2010). The responses of this tissue to noradrenaline support this conclusion, the predominant response being a β -adrenoceptor mediated relaxation and an inhibition of spontaneous phasic contractions (Moro et al., 2010). Responses to electrical stimulation in the present study were not affected by the adrenergic neurone blocker guanethidine at a concentration that almost abolished the neurogenic, sympathetic responses of the pig urethra.

Immunohistochemical studies of the bladder have identified the presence of a number of neuropeptides including tachykinins, enkephalin, calcitonin gene related peptide (CGRP), vasoactive intestinal peptide (VIP) and neuropeptide Y (Arms and Vizzard, 2011). Some of these have been associated with efferent nerves, while others are found in the afferent nerves within the lamina propria. Some of these neurotransmitters (eg. VIP) have relaxant effects on smooth muscle and are unlikely to be responsible for mediating urothelial/lamina propria contraction. It is interesting to note that neurokinin-A potently induces large contractions of this tissue which are mediated via the NK2 receptor (Sadananda et al., 2008). However, the NK2 antagonist GR159897, at concentrations that significantly antagonises contractions to exogenous neurokinin-A (Sadananda et al., 2008), had no effect on the responses to electrical field stimulation. A possible role for neuropeptide neurotransmitters was also investigated using inhibitors of endopeptidases. Peptidase enzymes located in the urothelium/lamina propria are important in terminating the effects of endogenous peptides, and inhibition of these enzymes will potentiate responses to peptides (Saban et al., 1997). Neither the neutral endopeptidase inhibitor phosphoramidon nor a commercially available cocktail of protease inhibitor with broad specificity for serine, cysteine, aspartic and aminopeptidases had any effect on responses to field stimulation. Pre-treatment of tissues with capsaicin to deplete neuronal stores of sensory neuropeptides also had no effect on responses of the urothelium/lamina propria to field stimulation. Furthermore, when capsaicin was added to the bath no contraction was observed suggesting that no contractile sensory neuropeptides were present, or at least no significant release occurs in these tissues.

In the detrusor muscle, NO is released as a co-transmitter with acetylcholine and inhibits smooth muscle contraction (Dokita et al., 1991). Thus NO synthase inhibition enhances responses to electrical field stimulation (Garcia-Pascual et al., 1991). In the

urothelium/lamina propria, NO donors relax the tissue and inhibit contractions to carbachol. However, the NO synthase inhibitor L-NNA has no effect on basal phasic contractions or those elicited by carbachol suggesting that the tissue is responsive to NO but that there is no basal release or muscarinic receptor stimulated release of NO in these tissues (Moro et al., 2012). In the present study the effects of L-NNA were examined on field stimulated tissues. The lack of effect following inhibition of NO synthase suggests that NO is not released as a co-transmitter in this tissue and does not influence nerve induced responses.

Only recently has the urothelium/lamina propria been shown to possess contractile properties and most studies examining bladder neurogenic responses do not state whether the urothelium/lamina propria was removed or intact. This may explain the variation in results reported in the literature. In human tissue for example, some studies have reported that EFS-induced bladder contractions are abolished by atropine (Sibley, 1984), while others have reported atropine-insensitive contractions (Sjogren et al., 1982, Tagliani et al., 1997). It is possible that the atropine-resistant contraction found in a number of studies across a range of species actually arose from the urothelium/lamina propria rather than the detrusor muscle itself.

7.6 CONCLUSIONS

This study has shown that electrical depolarisation of the nerves present in the porcine urothelium and lamina propria results in a frequency-dependent contraction of the tissue. This contraction is sensitive to tetrodotoxin, but resistant to inhibitors of adrenergic, cholinergic and purinergic neurotransmission. The identity of the neurotransmitter(s) has yet to be determined, but does not appear to be noradrenaline, acetylcholine or ATP and is unlikely to be a peptide.

Chapter 8

Effects of mechanical removal of the urothelium from pig bladder dome

Published abstracts and conference presentation arising from this chapter:

Moro, C., Sellers, D., Chess-Williams, R. The lamina propria is responsible for the generation of the spontaneous contractile activity of the urothelium/lamina propria. *Proceedings of the 45th Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists Scientific Meeting* Perth: 2011. Available at: <http://ascept2011.e proceedings.com.au/papers/p103.pdf>

8.1 INTRODUCTION

The urothelium plays a vital role as a barrier to the urine and dissolved substances in the bladder lumen. This tissue is a specialised surface lining the inside of the bladder and is composed of three cell layers: basal cells, intermediate cells, and the umbrella cells which cover the superficial apical surface (Figure 8-1). A range of mechanisms work together to assist in maintaining an effective urothelial barrier. For example, a coating of glycosaminoglycans and proteoglycans forms on the outer surface of the umbrella cells and protects the cells from dissolved solutes in the urine (Hurst and Zebrowski, 1994, Grist and Chakraborty, 1994, Nickel and Cornish, 1994). The apical surface of the umbrella cells also has a unique layer of plaques composed of uroplakins (Apodaca, 2004), a range of proteins expressed only in the urothelium, which form a unique and specialised barrier (Hu et al., 2005, Garcia-Espana et al., 2006). In addition, each cell in the urothelium maintains tight junctions between adjacent cells, composed of 4-6 interconnecting strands (Peter, 1978). These tight junctions form a physical barrier that is impermeable to solvents in the urine.

As discussed in previous chapters of this thesis, the urothelium releases several chemical mediators such as ATP, acetylcholine and nitric oxide (Ferguson et al., 1997, Cheng et al., 2011b, Munoz et al., 2011, Yoshida et al., 2008) which play a role in sensory signalling from the bladder (Birder, 2006). This wide range of properties identifies the urothelium as an important cell layer in the overall regulation of bladder activity. Previous chapters of this thesis have also shown the contractile activity of the urothelium/lamina propria. However, there is no clear evidence for a contractile-mediating cell type in the urothelium, suggesting that most of the contractile activity likely arises from the lamina propria. It is of interest to

identify whether the contractile properties of the bladder urothelium/lamina propria strips are influenced by the removal of the urothelium.

Prior methods for cell removal

Past studies have attempted to remove the inner lining from *in vitro* preparations of bladder while leaving the detrusor intact. Examples of this include both pig (Hawthorn et al., 2000) and human samples (Chaiyaprasithi et al., 2003, Kumar et al., 2004). However, the methods employed resulted in the removal of the urothelium *and* lamina propria, rather than just the urothelium. In order to assess the influence of removal of the urothelium on contractility of the urothelium/lamina propria a method is required that removes only the apical layers.

One method for removing only the endothelial cells from tissue preparations is by dabbing the surface with a paper towel, such as that used in blood vessels (Sade and Folkman, 1972). An alternative method involves gently rubbing the surface, as commonly used to remove the endothelium from blood vessels (Buchwalow et al., 2008). This study utilised a cotton-covered wire, although a variety of other tools have been used such as a wooden dowel (Moore et al., 2005) or other forms of mechanical debridement (Venugopalan et al., 1997, Baxter, 1995, Furchgott and Zawadzki, 1980). A recent study (Munoz et al., 2010) has utilised an adapted method in rat bladders, swiping the internal lining of the bladder with a cotton bud to remove the urothelium. Munoz *et al* (2010) appeared to demonstrate a method to remove the urothelium without specifically damaging the lamina propria. Alternatively, another study on pig bladder tissue removed only the urothelium by scraping the epithelial surface with a scalpel (Cheng et al., 2011b).

The present study aimed to identify which of the previously published methods is optimal for disrupting the majority of the porcine apical urothelial cells without damaging the underlying lamina propria. In addition, removing the urothelium may influence the contractile activity identified in intact urothelial/lamina propria strips. This was investigated by measurements of spontaneous activity and the responses to agonists, to distinguish if the pacemaker activity in the isolated tissue samples is inhibited following disruption to the urothelium.

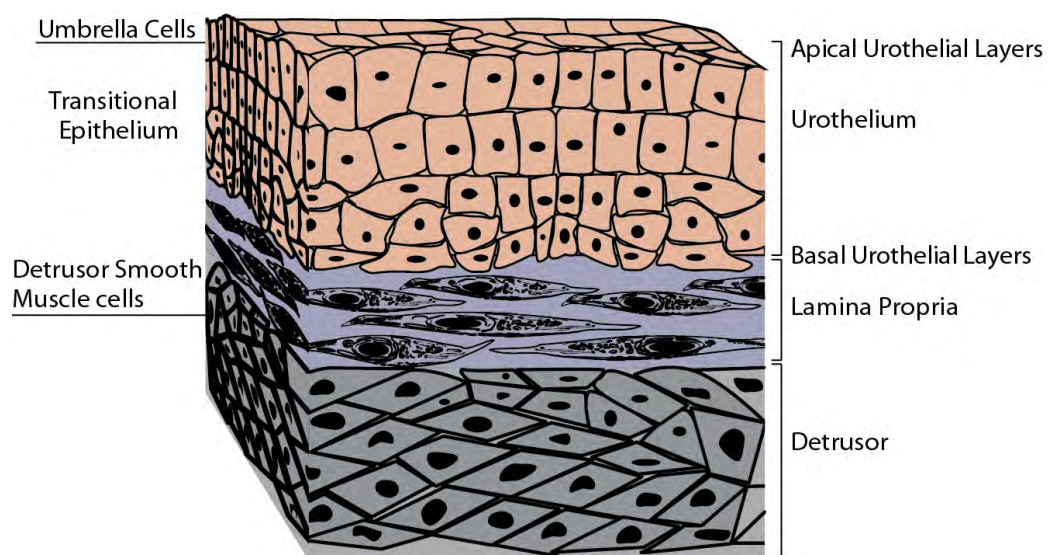


Figure 8-1: A schematic representation of a section of bladder wall illustrating the various cell layers.

8.2 METHODS

8.2.1 *Mechanical disruption of the urothelium*

Pig bladder was obtained and prepared as detailed in Chapter 2. Strips of urothelium/lamina propria were carefully dissected with scissors from the wall of the bladder dome. Several different methods were used to disrupt the urothelium and are detailed below.

1. Dabbing the tissue with a paper towel
2. A single longitudinal swipe with a cotton bud
3. Two longitudinal swipes with a cotton bud
4. Three longitudinal sweeps with a cotton bud
5. Four longitudinal sweeps with a cotton bud
6. A longitudinal scrape with the edge of a scalpel

8.2.2 *Histology*

Preparation of tissues for histology

Immediately after preparation of the bladder samples, the separate tissues were fixed in 10% Neutral Buffered Formalin (Fronine, QLD, AUS) and left for 24 hours. Following this, bladders were prepared for sectioning and staining by embedding in paraffin wax. This was performed using the following protocol:

- 75% Ethanol for 1 hr at 4°C
- 90% Ethanol for 1 hr at 4°C
- 100% Ethanol for 1 hr at 4°C
- 1:1 Xylene:ethanol for 30 minutes
- 100% Xylene for 30 minutes
- change of 100% Xylene, for 30 minutes

-
- 1: 1 Xylene:Paraplast bath, for 1 hour at 57°C
 - Liquid Paraplast at 57°C for 1 hour.
 - Change of liquid Paraplast at 57°C for 1 hour

Tissues were placed in aluminium rectangle moulds (Figure 8-2) and completely covered with liquid “Paraplast” tissue embedding medium (Oxford labware, St Louis, MO) using a Tissue-Tek TEC 111 Embedding Centre. These paraffin moulds were left to set overnight.

Serial sectioning

The wax sections were placed in a freezer until set and then removed from the aluminium boats. Strips of tissue embedded in paraffin were sectioned at 10 µm using a Reichert-Jung 2040 Autocut Microtome. Five sections across the tissue were sliced from each sample and placed on Starfrost microscope slides (ProSciTec, QLD, AUS) which were lightly coated with Mayers Egg Albumin. This was prepared by beating an egg white and leaving it overnight in a tall cylinder. Liquid from the bottom of the cylinder was pipetted out and added to an equal volume of glycerol. Sections were placed into heating racks at 57°C and left for 8 hours to dry prior to staining.



Figure 8-2: Rectangular moulds of paraffin holding the bladder specimens prior to sectioning and staining.

8.2.3 *Histological analysis*

Histology was performed using hematoxylin-eosin staining to determine the level of removal of the apical urothelial cells. Prior to staining with Haematoxylin and Eosin the paraffin was removed from the tissues using the following procedure:

- 100% Xylene, 1 minute
- 1: 1 Ethanol: Xylene, 3 minutes
- 100% Ethanol, 3 minutes
- 100% Ethanol, 3 minutes
- 90% Ethanol, 3 minutes
- 70% Ethanol, 3 minutes
- Distilled water, 5 minutes

The sections were stained using the following procedure:

- Mayers hematoxylin (Fronine, Qld), 10 minutes
- Scott's tap water, (blueing solution) 5 minutes
 - 4g Sodium hydrogen carbonate
 - 40g Magnesium sulphate
 - 2L Distilled water
- Acid alcohol, 1 minute
 - 29mL Distilled water
 - 1mL Concentrated HCL
 - 70mL ethanol
- Distilled water, 5 minutes
- Eosin phloxine solution (Fronine, Qld), 4 minutes
- Distilled water, 5 minutes

After staining the tissues underwent the following process to remove the stain from the tissue:

- 70% Ethanol bath, 3 minutes
- 90% Ethanol bath, 3 minutes
- 100% Ethanol bath, 3 minutes
- 100% Ethanol bath, 3 minutes
- 50% Ethanol 50% xylene bath, 3 minutes
- 100% Xylene, 3 minutes
- Left to dry overnight.

A coverslip was fixed to the slide with Biomount. Slides were then examined under a microscope for clarity in the image, and the individual layers of the bladder identified.

8.2.4 *Tissue Setup*

Strips of urothelium/lamina propria 2 cm long were mounted in Krebs-bicarbonate solution, as described in Chapter 2. The frequency of spontaneous contractions and baseline tension was recorded for control intact tissues and also those with a disrupted urothelium.

8.2.5 *Responses to agonists*

In separate experiments, samples of urothelium/lamina propria were dissected into 4 cm strips, and cut in half. The two 2 cm strips were set up in pairs, with one left as control (intact), and one swiped three times with a cotton bud to disrupt the urothelium. Frequency (cycles min^{-1}), and tension (g) responses were obtained in the absence and presence of carbachol (1 μM , $n = 8$) and isoprenaline (1 μM , $n = 8$). Responses to agonists were taken at the peak response and data obtained was analysed using paired Student's *t*-tests

8.3 RESULTS

Histological Studies

8.3.1 *Control Tissues*

In whole segments of bladder dome stained with hematoxylin-eosin, the urothelium, lamina propria and underlying detrusor were evident (Figure 8-3). Following removal of the detrusor muscle, the urothelium and lamina propria were not damaged from the dissection process (Figure 8-4). The umbrella cells were clearly intact and the process of preserving the tissues by fixation and embedding in paraffin wax was a successful method to retain the individual layers.

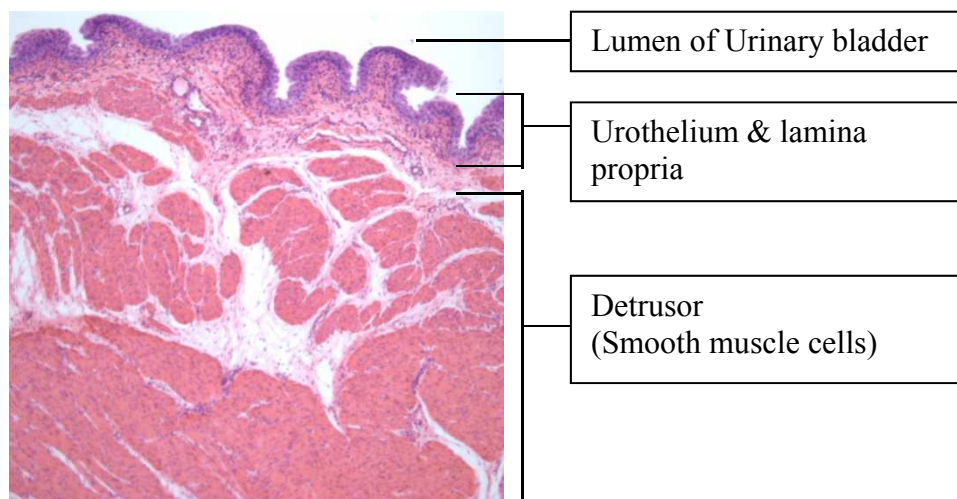


Figure 8-3: Transverse section of the bladder dome stained with haematoxylin and eosin. This control tissue was not denuded. The tissue beneath the urothelium and lamina propria is composed of detrusor smooth muscle (Magnification: 40x).

Mechanical disruption of the urothelium

8.3.2 *Effect of a single or double dab of the tissue with a paper towel*

An attempt was made to remove the urothelium using a method previously used on vascular endothelium, outlined by Sade and Folkman (1972). Tissues were dabbed with a “Kimwipe” paper towel one or two times. There was no observable difference between the urothelial cells of these tissues and those of controls (Figure 8-5, Figure 8-6).

8.3.3 *Effect of a single or double longitudinal swipe with a cotton bud*

Using an adapted method from Munoz *et al.* (2010), tissues underwent a single longitudinal swipe with a cotton bud. There was a very slight *compressing* of the urothelium with this method, although the apical cell layers were still intact and not removed from the tissue.

8.3.4 *Effect of a single or double longitudinal swipe with a cotton bud*

Two longitudinal swipes with a cotton bud was effective at removing some portions of the urothelium, although the success was inconsistent. There were also regions of the apical layer which were left relatively intact (Figure 8-7).

8.3.5 *Effect of three longitudinal sweeps with a cotton bud*

The effect of three longitudinal swipes with a cotton bud was also examined. In these samples a large proportion of the urothelium was removed down to the basal layers. This method produced a substantial disruption to the urothelium but left the lamina propria largely intact (Figure 8-8).

8.3.6 *Effect of four longitudinal swipes with a cotton bud*

Four swipes with cotton buds across the urothelial surface resulted in a clear and uniform disruption of the urothelium which was generally consistent throughout the samples (Figure 8-9). However, portions of the lamina propria were also disrupted, and large sections of the tissue underwent damage that affected the lamina propria.

8.3.7 *Effect of longitudinal scrape with the edge of a scalpel*

Scraping the tissue with a scalpel caused damage to both the urothelium and the lamina propria (Figure 8-10). Although the majority of damage was observed along the urothelium, in some sections the scalpel broke through the urothelium and damaged the lamina propria. In these sections the scalpel had dug directly into the lamina propria, causing severe damage to large numbers of cells within.

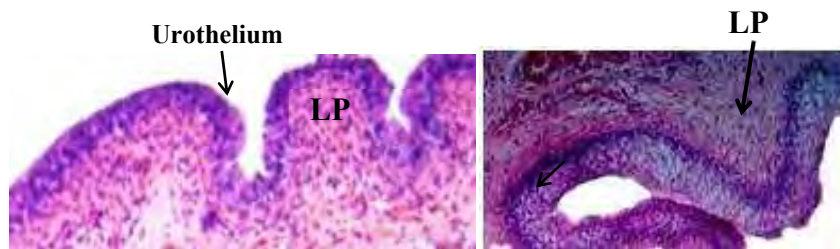


Figure 8-4: Control urothelium/lamina propria tissues after dissection from the detrusor muscle. The urothelium (arrows) and lamina propria (LP) were easily identifiable and not damaged by the dissecting procedure (Magnification: Left image: 100x. Right image: 400x).

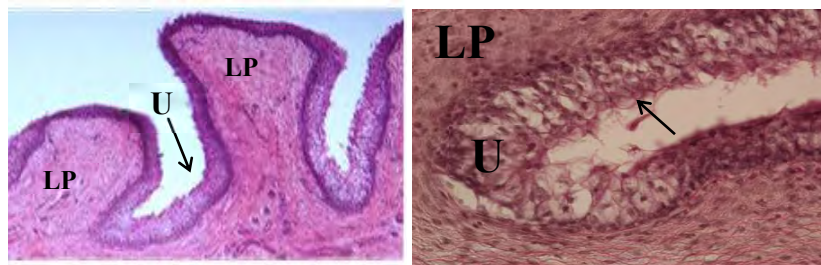


Figure 8-5: Effect of dabbing the tissue once with a paper towel. This method did not cause any clear disruption to the urothelium (U, arrows). Magnification: Left image: 100x. Right image: 400x

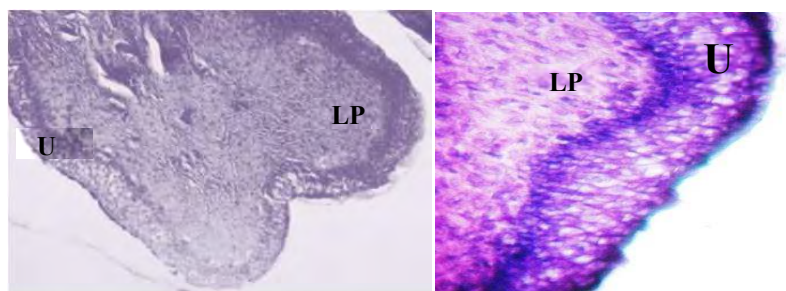


Figure 8-6: Effect of dabbing the tissue two times with a paper towel. Dabbing the tissue twice with a paper towel (Kimwipes) did not cause any clear disruption to the urothelium (Magnification: Left image: 100x. Right image: 400x).

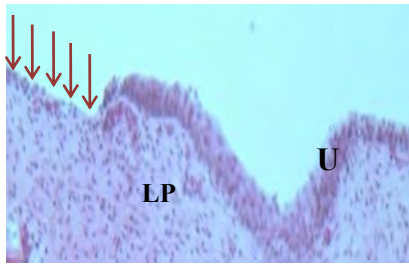


Figure 8-7: Effect of two longitudinal swipes with a cotton bud. Two swipes with cotton bud caused a very slight disruption to parts of urothelium (arrows) although the majority of the layer was kept intact (Magnification: Left image: 100x. Right image: 400x).

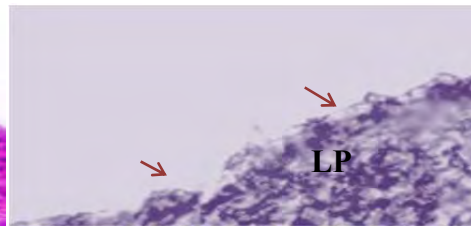
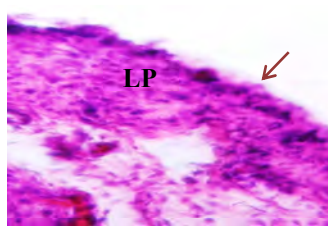


Figure 8-8: Effect of three longitudinal swipes with a cotton bud. Three swipes with cotton buds caused a clear and uniform disruption of the urothelium. This appeared consistent throughout the samples (Magnification: Left image: 100x. Right image: 400x).

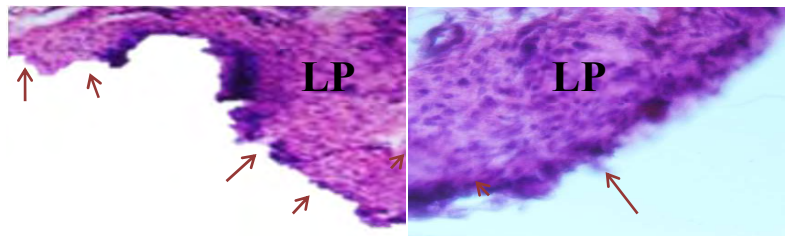


Figure 8-9: Effect of four longitudinal swipes with a cotton bud. Four swipes with cotton buds caused a clear and uniform disruption of the urothelium (arrows) which was generally consistent throughout the samples. However, portions of the lamina propria (LP) were also disrupted (Magnification: Left image: 100x. Right image: 400x).

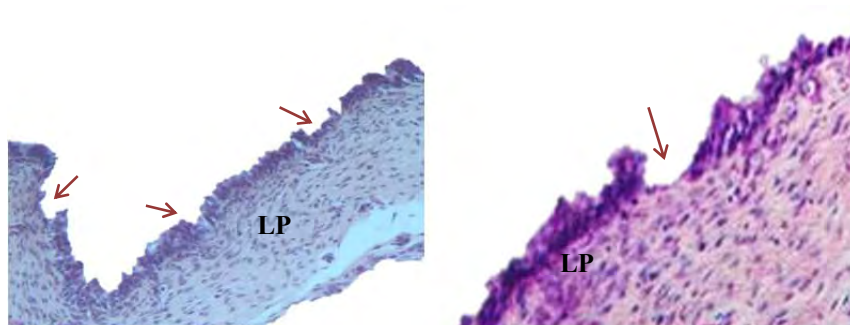


Figure 8-10: Effect of a longitudinal scrape with the edge of a scalpel. Scraping the tissue with a scalpel caused damage to the urothelium (arrows) and lamina propria (Magnification: Left image: 100x. Right image: 400x).

8.4 CONCLUSION FROM HISTOLOGICAL STUDIES

Upon histological analysis three swipes with a cotton bud was found to be the optimal method to successfully disrupt the urothelium without damaging the lamina propria. This method was used for subsequent functional studies.

8.4.1 *Spontaneous activity of urothelium/lamina propria strips following removal of the urothelium*

There was no significant difference in the frequency of spontaneous activity or baseline tension between the control tissues and those that underwent urothelium removal with three swipes of a cotton bud (3.06 ± 0.22 cycles min^{-1} vs 2.99 ± 0.26 cycles min^{-1} for frequency, $1.57 \pm 0.18\text{g}$ vs $1.57 \pm 0.16\text{g}$ for tension, $n = 8$).

8.4.2 *Response to carbachol*

After a 60 minute equilibration, responses were obtained to carbachol (1 μ M, n = 8). The increase in contractile frequency induced by carbachol was similar between the control (Figure 8-11) and urothelium-denuded tissues (2.02 ± 0.80 cycles min^{-1} vs 1.55 ± 0.32 cycles min^{-1} , Figure 8-12). The increase in basal tension to carbachol was also similar in the two groups of tissues (3.27 ± 0.37 g vs 1.57 ± 0.16 g, Figure 8-13).

8.4.3 *Response to isoprenaline*

Isoprenaline (1 μ M, n = 8) had a clear effect on the bladder, causing strong relaxations of the tissue. The decrease in contractile frequency induced by isoprenaline was similar in control and urothelium-denuded tissues (0.88 ± 0.18 cycles min^{-1} vs 1.15 ± 0.22 cycles min^{-1}). In addition, the decrease in basal tension to isoprenaline was not significantly different between the two groups of tissues (1.01 ± 0.07 g vs 1.35 ± 0.16 g, Figure 8-14)

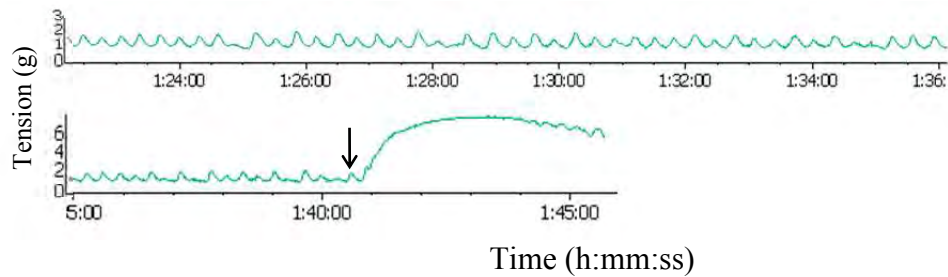


Figure 8-11: Representative traces depicting the baseline spontaneous contractile activity (top image) and the response to carbachol (at arrow, 1 μ M, bottom image) in a control tissue

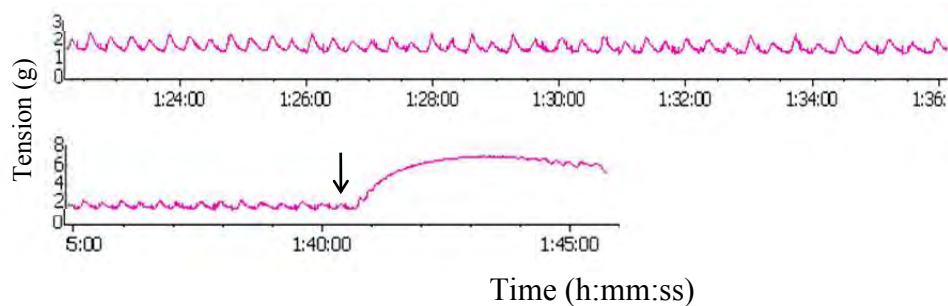


Figure 8-12: Representative traces depicting the baseline spontaneous contractile activity (top image) and the response to carbachol (at arrow, 1 μ M, bottom image) in a tissue which had undergone longitudinal swiping three times with a cotton bud.

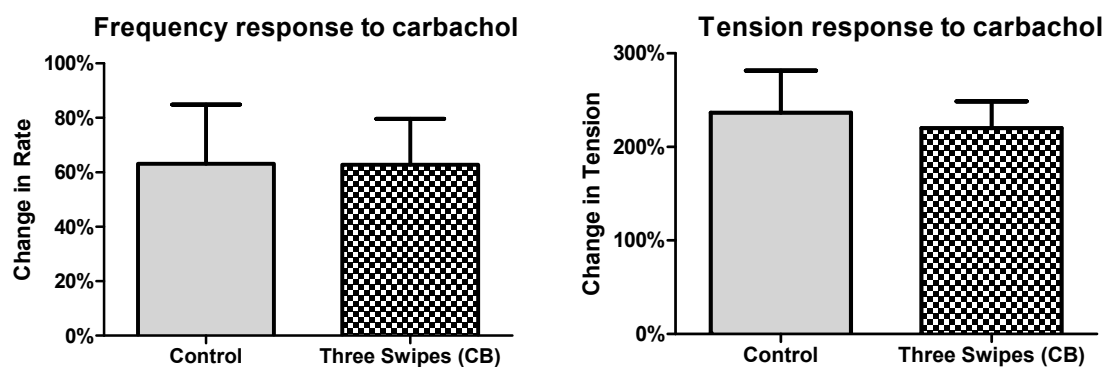


Figure 8-13: The effect of carbachol ($1 \mu\text{M}$, $n = 8$) to frequency or tension on tissues that had undergone three swipes with a cotton bud (CB) compared to controls. There was no significant difference in the spontaneous contractile frequency or the tension response to carbachol.

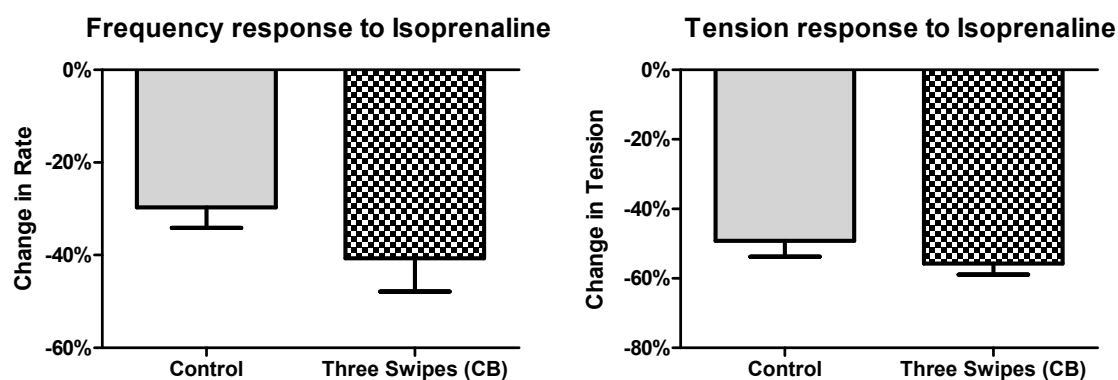


Figure 8-14: The effects of isoprenaline ($1 \mu\text{M}$, $n = 8$) to frequency or tension on the tissues after three swipes with a cotton bud (CB) as above. There was no significant difference in the spontaneous contractile frequency or the relaxation responses to isoprenaline.

8.5 DISCUSSION

Isolated strips of the urothelium and lamina propria develop spontaneous contractions which can be modulated via activation of the muscarinic and adrenergic receptor systems as discussed in previous chapters. However, the mechanisms underlying this activity are unknown and it is not clear how the urothelium influences the overall activity of the bladder. It is likely that the cells involved in the generation, coordination and contractile function of the spontaneous activity lie within in the lamina propria rather than in the urothelium. Although still not proven, this hypothesis has been suggested by authors of prior studies examining this layer (Ikeda et al., 2007, Cheng et al., 2011b, Fry et al., 2012).

It is known that removal of the urothelium/lamina propria affects detrusor contractions. This has been shown in a study where contractile responses to electrical field stimulation in the feline bladder were significantly increased following urothelium/lamina propria removal (Levin et al., 1995). Additionally, isolated pig bladder strips also demonstrated increased contractile responses to muscarinic receptor stimulation after urothelium/lamina propria removal, and this is thought to be due to an unknown urothelium derived inhibitory factor (Templeman et al., 2002b, Hawthorn et al., 2000). Removal of the urothelium from rat bladder strips does not appear to affect basal release of nitric oxide or ATP (Munoz et al., 2010). However, in that study urothelial removal did produce a significant reduction in carbachol-induced release of nitric oxide and electrical-field stimulated release of ATP. It was unclear whether these results related functionally to the spontaneous contractile activity of urothelium/lamina propria or solely on the contractile ability of the detrusor smooth muscle.

In an attempt to disrupt the urothelium while leaving the lamina propria intact, previously published methods for removal of this cell layer were investigated. Histological examination showed that one or two dabs with a paper towel had no effect on the urothelium. Although past studies have used this method successfully in vascular endothelium (Sade and Folkman, 1972), it does not appear to influence the bladder urothelium to the same effect. This is most likely due to the presence of a squamous epithelium in blood vessels compared to a transitional epithelium on the urothelium.

Scraping with a scalpel, which has been used previously on pig bladders (Cheng et al., 2011b), did effectively disrupt the urothelium. However, this method appeared to ‘tear’ away sections of the lamina propria. It is possible that careful use of a scalpel blade, giving consideration to the angle and direction of the blade, would be an effective method for collection of urothelial cells or removing the urothelium from small areas of the tissue.

Mechanical methods for removing the apical layers via swabbing or rubbing the epithelial surface have also been used successfully in a range of tissues (Furchgott and Zawadzki, 1980, Buchwalow et al., 2008). Additionally, longitudinal swiping has been successful in rat urinary bladder (Munoz et al., 2010). One or two swipes with a cotton bud were ineffective at removing large proportions of urothelium from the luminal surface in the present study. This may differ from that experienced in vascular studies, due to the larger and potentially more robust nature of the porcine bladder urothelium in comparison to the endothelium in rat bladders or blood vessels. However, three longitudinal swipes with a cotton bud consistently disrupted the urothelial layer across the tissue samples. This method was less invasive and damaging to the lamina propria than scraping with a scalpel. After this success, four swipes

with a cotton bud were attempted, but this method started to impact the underlying lamina propria.

Upon confirming the validity of the removal method, paired functional experiments were performed to identify the impact of three swipes with a cotton bud on the lamina propria contractile activity. The basal spontaneous frequency observed in previous chapters was still evident and not altered by urothelium removal. In addition, responses to muscarinic receptor stimulation or β -adrenoceptor stimulation were also not affected by urothelial removal. This suggests that the urothelium is not involved in mediating the spontaneous contractile activity of the urothelium/lamina propria. This was interesting, as urothelial cells are known to express a range of receptors such as muscarinic (Wang et al., 1995), vanilloid, and adrenergic receptors (Birder et al., 2001). Additionally, the urothelium can release a number of chemical mediators including acetylcholine (Yoshida et al., 2006); nitric oxide (Birder et al., 1998); and ATP (Ferguson et al., 1997). Although some of these, such as ATP, can also be released from the urothelium and lamina propria (Sadananda et al., 2012, Sadananda et al., 2009), it was interesting to identify a lack of effect after removal of the urothelium.

A diverse collection of cells reside within the lamina propria. These include connective tissue, blood vessels, and afferent nerve fibres; as well as a range of cells types which include interstitial cells and fibroblasts (Johnston et al., 2010, McCloskey, 2010, Rasmussen et al., 2009, Wiseman et al., 2003, Yu et al., 2011b, Woodman et al., 2011). Additionally, a variety of immune cells are also present in the lamina propria, such as mast cells and dendritic cells (Christmas and Rode, 1991, Gardiner et al., 1986, Kummer et al., 2007, Yu and Hill, 2011). Within this layer, the interstitial cells most likely mediate contractile activity and the responses to receptor activation. These cells have been discussed in Chapter 1, and it is

understood that they have the ability to contract and hold close associations with the calcium transients and spontaneous activity (Fry et al., 2007, Sui et al., 2008, Fry et al., 2012). Additionally, there is a possibility that these types of cells are involved in contractile activity during wound healing responses (Gabbiani, 2003). Interstitial cells have also previously been suggested as the cell type mediating urothelial/lamina propria contractions in response to neurokin-A (Sadananda et al., 2008). Therefore, it appears from past research that the interstitial cells are likely modulators for the observed responses demonstrated in strips of urothelium-removed lamina propria.

In conclusion, disruption to the apical urothelial cells of the pig bladder could be accomplished by a swipe with a scalpel and swiping three times with a cotton-bud, but not by dabbing with a paper towel. The optimal method for urothelium removal was determined to be three swipes with a cotton bud. Disruption to this layer did not affect the rate of spontaneous contractions, the response to muscarinic receptor stimulation or the response to β -adrenoceptor activation of the underlying tissue. This indicates that the phasic activity of the urothelium/lamina propria most likely arises from cells within the lamina propria and is independent of urothelial regulation.



Chapter 9

GENERAL DISCUSSION

The urothelium/lamina propria is known to play an important role in the maintenance of normal bladder function via the release of chemical mediators. In addition, the urothelium/lamina propria exhibits spontaneous contractile activity and this may influence contractions within the bladder. This thesis investigated the factors mediating contractile activity of the bladder urothelium/lamina propria.

9.1.1 The spontaneous activity of the urothelium/lamina propria

An important part of this study was the clear classification of the urothelial/lamina propria spontaneous contractile activity. This phasic activity occurs at a rate of 3 - 4 cycles per minute and develops spontaneously *in vitro* in urothelial/lamina propria tissue strips. The ability of the urothelium/lamina propria to contract has been demonstrated previously in a range of studies inducing contraction in: the lamina propria from rabbit urethra after EFS or exogenous noradrenaline (Mattiasson et al., 1985a, Zygmunt et al., 1993); urothelium/lamina propria from rat bladder to the P2Y agonists ADP, UTP and UDP (Fry et al., 2012); guinea pig urothelium/lamina propria to EFS (Heppner et al., 2011); and pig urothelium/lamina propria to neurokinin A or carbachol (Sadananda et al., 2008). The present study has further characterised this contractile activity.

In an attempt to elucidate the underlying mechanisms for the urothelium/lamina propria spontaneous contractile activity a number of investigations were completed. Although the urothelium/lamina propria receives a rich density of sensory and parasympathetic nerve fibres (Birder, 2011, Dickson et al., 2006), its spontaneous contractile activity was insensitive to tetrodotoxin in the present study, suggesting a non-neuronal mechanism of generation. This was supported by EFS experiments, which showed that although nerve depolarisation could

evoke contractions, the frequency of spontaneous activity remained unaltered. In addition, L-NNA had no effect on the contractile response to EFS or the spontaneous contractile frequency and basal tension. This finding for the urothelium/lamina propria was interesting, as neuronally-derived nitric oxide is thought to modulate spontaneous activity in detrusor smooth muscle, and as such, was considered a potential regulator for the urothelium/lamina propria (Meng et al., 2012). However, although NOS-inhibition had no effect on spontaneous activity, the addition of NO donors did cause a relaxation in tension and decrease in contractile frequency in the urothelium/lamina propria. These results suggest that this tissue can respond to nitric oxide through mechanisms within the urothelium/lamina propria, although NO is not released endogenously, as previously suggested by Munoz *et al.* (2010). Prostaglandins were also considered to be potential mediators of urothelial/lamina propria activity, and are known to have cytoprotective functions within the gut mucosa and possibly in the urinary bladder (Milman, 2007). However, in this study the spontaneous contractile frequency, basal tension or response to EFS were unaffected by the application of indomethacin.

In the absence of any agonist, the spontaneous contractions of the urothelium/lamina propria were also unaltered following antagonism of muscarinic, purinergic, or adrenergic receptors, even though these receptors are known to be functionally active in the urothelium/lamina propria (Birder, 2011). Neuropeptides are potential mediators of lower urinary tract function (Arms and Vizzard, 2011), yet the spontaneous contractile activity or responses to EFS were unaffected by the administration of antagonists for common neuropeptides. Additionally, no effect was seen after the administration of capsaicin, which causes the release of neuropeptides (Szolcsanyi, 2004). This lack of response is consistent with a recent study which identified that capsaicin was not involved in stimulating either contraction or release of

ATP from pig urothelium/lamina propria (Sadananda et al., 2012). Also, an NK2 receptor antagonist had no effect on responses to EFS or on basal contractile activity, even though exogenous neurokinin-A is known to cause contraction (Sadananda et al., 2008). Although these receptor antagonists do not affect urothelial/lamina propria activity or the responses to EFS, receptor activation did modulate the spontaneous contractions indicating that these receptors and systems may be important in regulation of frequency or generation of spontaneous contractile activity.

9.1.2 Spontaneous contractile activity in the detrusor

The urothelium/lamina propria may be involved in regulating the detrusor, where a degree of spontaneous contractile activity is also present (Sibley, 1984, Fry et al., 2010). These detrusor contractions arise via myogenic spontaneous activity present in the detrusor (Levin et al., 1986) due to increases in the levels of sarcoplasmic calcium (Wu et al., 1995). This increase can occur due to an influx of extracellular calcium via L-type or T-type channels (Montgomery et al., 1992, Sui et al., 2003), or by release from intracellular stores (Wu et al., 2002, Fry et al., 1994). This calcium influx initiates the detrusor contractions, which are followed by potassium-mediated repolarisations (Hashitani and Brading, 2003, Herrera et al., 2000, Heppner et al., 1997, Young et al., 2008, Thorneloe and Nelson, 2003). These processes result in contraction-relaxation phasic cycles.

Detrusor spontaneous contractions during the filling stage occur during detrusor overactivity in both human (Mills et al., 2000) and pig bladder models of detrusor overactivity (Milicic et al., 2006) and this may be due to enhanced calcium oscillations in the detrusor muscle layers (Sui et al., 2009). These oscillations are not unique to the detrusor and it is known that

calcium signals collectively regulate a variety of functions in muscle cells, including contractility, modulation of membrane potential and regulation of gene expression (Hill-Eubanks et al., 2011).

9.1.3 The influence of the urothelium/lamina propria on bladder activity

It is evident that spontaneous activity within the urothelium/lamina propria likely influence bladder activity. This has been demonstrated in a number of studies in animal models. Firstly, the presence of the urothelial/lamina propria enhances the spontaneous contractile activity in intact bladders (Ikeda and Kanai, 2008, Sui et al., 2008). Secondly, intact tissues generate larger propagating calcium waves in the detrusor than that of denuded samples (Ikeda and Kanai, 2008, Sui et al., 2008, Fry et al., 2012). Thirdly, calcium mapping techniques have shown that spontaneous calcium waves in rat bladders were generated from within the lamina propria and then spread outward towards the urothelium and detrusor (Ikeda et al., 2007, Kanai et al., 2007). The phasic calcium transients were linked to spontaneous contractile activity in rats, and this activity was augmented by ADP, UTP and UDP, the specific purinergic agonists which stimulate P2Y receptors on lamina propria interstitial cells (Fry et al., 2012). Fry *et al* (2012) suggested that these interstitial cells were instrumental in determining the contractile properties of bladders that exhibited overactivity.

In the present study, the importance of calcium channels in regulating the urothelial/lamina propria contractile activity was further supported by the addition of nifedipine (1 μ M, n = 8), which significantly inhibited the frequency ($P < 0.05$) and tension ($P < 0.01$) of contractions. This finding is consistent with a recent study which demonstrated that nifedipine abolished both the spontaneous contractions of the urothelium/lamina propria, and calcium transients in

guinea-pig bladders (Heppner et al., 2011). Therefore, these results further suggest the involvement of calcium influxes from the urothelium/lamina propria in influencing bladder contractions.

9.1.4 The role of interstitial cells within the lamina propria as the generators of contractile activity

In the present study the importance of the lamina propria in regulating bladder spontaneous activity was investigated by debridement of the urothelium. Disruption to the apical urothelial layer did not affect the rate of spontaneous contractions nor the responses to muscarinic receptor stimulation or β -adrenoceptor activation. This thereby identified the lamina propria as the primary layer generating the spontaneous activity, although which cell type involved has not yet been determined. However, it is likely to arise from interstitial cells within this layer.

In the gastrointestinal tract interstitial cells have been widely researched and are understood to act as pacemaker cells, stretch receptors and intermediaries between nerve and smooth muscle cells (Sanders and Ward, 2006). Throughout the lower urinary tract, such as in the prostate, interstitial cells are known to generate spontaneous calcium transients, yet these have not always been linked to contraction or pacemaker potential generation (Lam et al., 2011). Since the identification of interstitial cells in the bladder (Smet et al., 1996b) a number of hypotheses have been proposed implicating these cells in the maintenance of normal bladder function. Interstitial cells form a network within the lamina propria (McCloskey, 2011), and may have a role in mediating the spontaneous activity of the bladder (Wiseman et al., 2003, Fry et al., 2004a, Gillespie et al., 2006b). Lamina propria interstitial

cells not only elicit spontaneous electrical and calcium events but also respond to exogenous agonists such as ATP (Fry et al., 2007). Interestingly, they appear responsible for the increased spontaneous calcium transients and contractile activity observed in the bladders of spinal cord transected mice (Ikeda and Kanai, 2008), a suggestion supported by reports of increased interstitial cell density within the lamina propria of experimental models of detrusor overactivity (associated with bladder outlet obstruction) in both rat (Kim et al., 2011) and guinea pig (Kubota et al., 2008). Detrusor overactivity has shown to be associated with increased numbers of interstitial cells in human detrusor (Biers et al., 2006b), although this study only examined one patient with idiopathic detrusor overactivity and one with neurogenic detrusor overactivity. Additionally, increases in gap junction expression between interstitial cells, demonstrated with connexin-43 staining, has been observed in human (Roosen et al., 2009) and rat (Ikeda et al., 2007) neurogenic overactive bladders, and morphological changes in these cells are also seen in patients with this condition (Gevaert et al., 2011).

9.1.5 Modulation of urothelial/lamina propria activity

Increases in urothelial/lamina propria spontaneous activity

During urodynamic observations, patients with overactive bladder who have unexpected spontaneous contractions during the filling stage can be diagnosed with detrusor overactivity (Al-Ghazo et al., 2011, Abrams et al., 2002). The mechanisms underlying this bladder disorder are not known, but the fact that the urothelium/lamina propria develops spontaneous contractions suggests a potential influence. Therefore, mechanisms that result in increases to frequency or tension of the urothelium/lamina propria activity warrant investigation.

In the detrusor smooth muscle, stimulation of muscarinic M₃-receptors is known to result in contractions (Fetscher et al., 2002, Chess-Williams, 2002a). On the urothelium/lamina propria the nicotinic receptor agonist DMPP did not affect contractile activity. However, muscarinic receptor agonists did increase the rate and basal tension of spontaneous contractions via the M₃ muscarinic receptor. Also, after tissue distension the spontaneous contractile frequency of the urothelium/lamina propria was increased. This increase in frequency was not mediated via ATP, NO or prostaglandins, but via acetylcholine acting via the M₃-receptors. The finding that stretch activates M₃-muscarinic receptors within the urothelium/lamina propria and thus increases the rate of contractions implicates these receptors with a potential role in modulating the spontaneous activity.

The additional finding that stretch-induced increases in spontaneous contractile rate were insensitive to tetrodotoxin supports the idea that during the storage phase of micturition, acetylcholine is released from the urothelial cells rather than nerves. This links to current research, whereby human bladder strips release increased non-neuronal acetylcholine during stretch, which is significantly greater with the presence of the urothelium/lamina propria (Yoshida et al., 2004b), and also enhanced with age (Yoshida et al., 2006). This has not solely been observed in tissue experiments, as non-neuronal acetylcholine is also released during hypotonic stretching of cultured urothelial cells (Hanna-Mitchell et al., 2007). This stretch-induced acetylcholine release likely influences detrusor function. Electrophysiological studies in rats have shown that responses to both carbachol and stretch commence in the urothelial layers, whereby the calcium and membrane potential transients begin near the urothelial-lamina propria interface before spreading to the detrusor (Kanai et al., 2007). This suggests that stimulation of the muscarinic receptors in the

urothelium/lamina propria during stretch possibly contributes to calcium-mediated spontaneous contractions in the detrusor.

Within the lamina propria the M₃-muscarinic receptor subtype is present on interstitial cells, as demonstrated in immunohistochemical studies (Grol et al., 2009). An additional importance for these lamina propria M₃-receptors is suggested by their upregulation in obstructed guinea pig bladders (Grol et al., 2011). This increased receptor density may present a mechanism that underlies the increased sensitivity to muscarinic stimuli from cholinergic agonists, observed in guinea pig models of bladder obstruction (de Jongh et al., 2007a). Additionally, an increased density of urothelial/lamina propria M₂ and M₃ muscarinic receptors is associated with overactive bladder in humans (Mukerji et al., 2006b) and in a bladder outlet obstruction models in rats (Kim et al., 2008), further implicating a role for these receptors in pathological states.

In the present study the predominant response to noradrenaline in the urothelium/lamina propria was relaxation via the β -adrenoceptors, however, mRNA was found to be present for all α -adrenoceptor subtypes. Surprisingly, in the presence of propanolol, the addition of exogenous noradrenaline resulted in significant increases to frequency and tension in strips of urothelium/lamina propria. This effect was also seen with α_1 -adrenoceptor agonists, which increased the spontaneous contractile frequency and tension via the $\alpha_{1A/L}$ -adrenoceptor subtype. Interestingly tamsulosin, an α_1 -adrenoceptor antagonist used clinically, was able to depress these increases in frequency and tension at low concentrations. α_1 -adrenoceptor antagonists have been successfully used for many years as treatments for benign prostate hyperplasia (Lepor et al., 2012, Nickel, 2003). The main target for their therapeutic action is the α_{1A} -adrenoceptor mediated contraction of smooth muscle in the prostate which constricts

the urethra (Ventura et al., 2011). However, new clinical evidence suggests that α_1 -adrenoceptor antagonists may also act on other areas of the lower urinary tract and potentially alleviate lower urinary tract symptoms, such as urgency, through mechanisms independent of prostatic smooth muscle tone (Yoshida et al., 2012, Barendrecht et al., 2008, Chess-Williams, 2002b). It is not clear which regions in the bladder contain functionally active α -adrenoceptors, although there is data supporting mRNA and protein expression of these receptors in the rat urothelium (Yanase et al., 2008, Ishihama et al., 2006) and human lamina propria (Kurizaki et al., 2011). Two studies identified decreases in maximum micturition pressure during urodynamic analyses in patients administered silodosin, an α_{1A} -selective adrenoceptor antagonist (Yamanishi et al., 2010, Matsukawa et al., 2009). The effect of these drugs has been further demonstrated in a rat model, where tamsulosin increases urine flow but also reduces bladder overactivity (Okutsu et al., 2011). There is a scarcity of functionally active postjunctional α_1 -adrenoceptors located on the detrusor muscle (Goepel et al., 1997, Michel and Vrydag, 2006, Yamada and Ito, 2011), and as such, the data presented in this thesis suggests that the clinical α_1 -adrenoceptor antagonists might act on receptors within the urothelium/lamina propria to alleviate symptoms of overactive bladder. This potential for these drugs is of considerable interest and may provide future pharmacological treatments.

There remain a range of other receptor systems within the urothelium and lamina propria that when stimulated, increase contractile activity and hence warrant future research. The urothelium/lamina propria is known to contract in response to stimulation of neurokinin-2 receptors (Sadananda et al., 2008), and both frequency and tension were increased following administration of neurokinin-A (Chapter 7). Histamine also caused an increase in baseline tension of $15 \pm 2\%$ ($10 \mu\text{M}$, $n = 12$, $p < 0.001$) although it did not increase the frequency of contractions. Excessive angiotensin II activity has been suggested as a contributing factor for

lower urinary tract symptoms (Comiter, 2012), and an increase in tension and frequency was observed in the present study upon addition of exogenous angiotensin II (100 nM, $P < 0.01$, $n = 8$). The additional increases in frequency and tension observed with prostaglandin E2 ($P < 0.01$, $n = 8$) are also consistent with a recent study demonstrating its effect on enhancing spontaneous activity in the mouse urinary bladder (Kobayter et al., 2012). Past research has identified prostaglandin release in both human (Abrams et al., 1979, Jeremy et al., 1987, Mikhailidis et al., 1987) and dog (Gilmore and Vane, 1971) bladder samples, and although application of indomethacin had no effect on the urothelium/lamina propria basal spontaneous contractile activity in the present study, abnormal prostaglandin release may contribute to lower urinary tract symptoms. Thus, drugs targeting NK2, angiotensin II or prostaglandin E2 receptors may potentially be of clinical use in pathologies of the bladder.

Decreases in urothelial/lamina propria spontaneous activity

Mechanisms within the bladder which reduce the frequency of spontaneous contractions present novel therapeutic targets which may alleviate some symptoms of bladder pathology. This is evident with the range of new β -adrenoceptor agonists currently undergoing successful clinical trials as therapeutic treatments for overactive bladder (Gulur and Drake, 2010, Gras, 2012). In the urothelium/lamina propria, the predominant response to noradrenaline was found to be a relaxation and inhibition of spontaneous contractile activity via β -adrenoceptors. This relaxation to noradrenaline is enhanced following antagonism of the α -adrenoceptors. Inhibitory effects on the urothelium/lamina propria were also observed with other β -adrenoceptor agonists, and mediated predominantly via β_2 -adrenoceptors.

The urothelium/lamina propria was sensitive to NO which reduced the tension and slowed the rate of spontaneous activity. However, there did not appear to be any spontaneous NO release or muscarinic receptor-mediated NO release. The beneficial effects of administration of NO have been investigated previously, whereby oral nitrates were found to improve peak urinary flow rates and reduce residual urine volume in patients with bladder outlet obstruction (Klotz et al., 1999). However, there was no significant change to micturition parameters in asymptomatic patients and although the effectiveness of NO is still uncertain the present study implicates the urothelium/lamina propria as a target for future trials of such agents.

9.1.6 The physiological role of the urothelial/lamina propria contractile activity

It is unknown exactly why the bladders of many species exhibit spontaneous contractions, yet a range of hypotheses have been presented since their initial discovery in humans, pigs and rabbit bladders (Sibley, 1984). These contractions may stimulate nerves in the lamina propria (Gillespie, 2004a); maintain muscular tone (Fry et al., 2010); allow the individual muscle bundles to adjust their length in response to filling (Brading, 2006); or directly influence the detrusor to maintain normal continence during the filling stage.

Another hypothesis put forward in this thesis is that an additional action of the spontaneous urothelial/lamina propria contractions might be to maintain tight junctions between the umbrella cells in the apical urothelial layer. As the bladder fills the urothelium stretches to allow for a greater surface area, to accommodate an increasing volume of urine. The interstitial cells under the urothelium might contract in order to pull together the cells on the upper surface, keeping them tightly connected during stretching. Throughout bladder filling,

urothelial cells could release acetylcholine to act on M₃ receptors and stimulate faster lamina propria contractions, pulling the urothelial cells together and assisting with the maintenance of a tight apical-layer junction. Thus, the urothelial/lamina propria spontaneous contractile activity could act as an additional protective system which responds to the intensity of stretch and thus prevents the apical cell layer from reducing its barrier function or overstretching. Maintaining consistently tight and stable connections between umbrella cells throughout the filling stage would also contribute to the preservation of the uroplakin and GAG layers which coat the apical surface. It would be of great interest to investigate these various hypotheses for the roles of spontaneous urothelial/lamina propria contractions in future studies.

9.1.7 Implications for future drug development and future directions for the research described in this thesis

The muscarinic receptor-mediated increases to the spontaneous contractile activity of the urothelium/lamina propria may have important clinical implications. Muscarinic antagonists are the most common treatments for bladder overactivity (Sellers and Chess-Williams, 2012) and drugs used clinically to alleviate the symptoms of overactive bladder: darifenacin, oxybutynin, tolterodine and solifenacin, were used on the urothelium/lamina propria to identify their effects in mediating frequency and tension increases to carbachol. All four drugs were able to depress muscarinic receptor-mediated and stretch-induced increases in spontaneous contractile activity. The antagonism by these clinical antimuscarinics at the muscarinic receptors on the urothelium/lamina propria, which has a higher density of muscarinic receptors compared to the detrusor (Hawthorn et al., 2000), suggests an additional mechanism of action of these agents when alleviating symptoms associated with overactive bladder.

There was a higher contractile frequency in the urothelial/lamina propria strips compared to intact-bladder or detrusor strips, suggesting a limited influence of the urothelial/lamina propria in regulating normal bladder contractions. Although the link between *in vitro* urothelial/lamina propria contractions and *in vivo* whole-bladder contractions has never been ascertained, the urothelial activity may be more tightly coupled in diseased states of the bladder, where increased rates and amplitudes of contractions during the filling stage are associated with overactive bladder (Al-Ghazo et al., 2011). It has been demonstrated in rat bladders that calcium transients arise in the urothelium before propagating to the detrusor (Kanai et al., 2007). This urothelial spontaneous activity and its potential to spread to the detrusor layer (Fry et al., 2007) suggests that abnormal rates of spontaneous activity shown in some bladder diseases may be generated from the urothelium/lamina propria. This would result with an increased spontaneous activity in the detrusor during filling, such as that observed by urodynamics in sufferers of detrusor overactivity.

Further investigation into the functional roles of the spontaneous activity in the urothelial/lamina propria and the potential increased coupling to detrusor contractions in disease will provide a better understanding of the underlying causes in bladder malfunction. As a target for pharmacological treatment, the urothelium/lamina propria presents a novel site which responds to receptor stimulation to modulate and regulate spontaneous activity. If malfunction of this tissue presents as a cause or mediator of bladder overactivity, its functionally active receptors and activity may be selectively targeted in treatments.

9.1.8 *Final remarks*

This thesis has identified the receptor systems modulating the spontaneous contractile activity of the urothelium/lamina propria. It is clear that the urothelium/lamina propria plays important roles in the maintenance of normal bladder function. A greater understanding of the functionally active receptors and systems in this tissue will enable novel therapeutic targets to be identified and investigated. Future avenues of research may further identify the relationships between these systems in the normal and pathological states of the human bladder.



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